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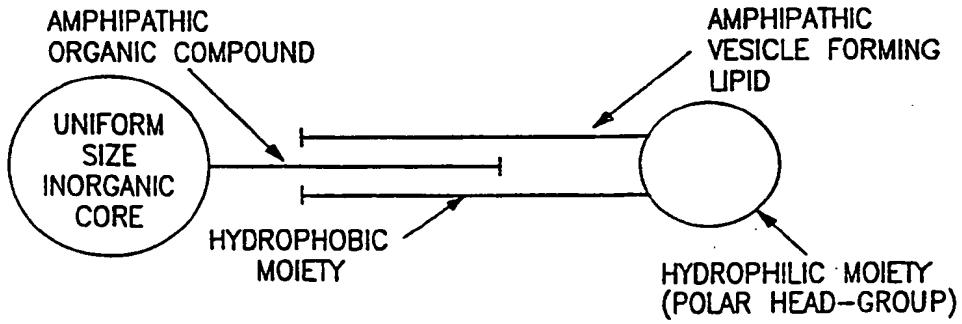

(57) Abstract

Inorganic oxides of substantially uniform particle size distribution are prepared by contacting aqueous solutions of an inorganic salt and an inorganic base across a porous membrane (14) wherein the membrane contains a plurality of pores which allows for precipitation of a substantially mono-dispersed size inorganic oxide particles on one side of the membrane and precipitation of a salt of the corresponding base on a second side of the membrane (Fig. 1). The particles so prepared can be coated with an organo-metallic polymer having attached thereto an organic functionality to which a variety of organic and/or biological molecules can be coupled. Particles so coupled may be used for in vitro or in vivo systems involving separations steps or the directed movement of coupled molecules to particular sites, including immunological assays, other biological assays, biochemical or enzymatic reactions, affinity chromatographic purification, cell sorting and diagnostic and therapeutic uses. In a further embodiment, described herein are liposome compositions which comprise the substantially uniform size inorganic core coated with an amphipathic organic compound and further coated with a second amphipathic vesicle forming lipid (Fig. 2). Also disclosed are novel phenyl lipid compounds which serve as the vesicle forming lipid (Fig. 3). When the magnetic particles are electromagnetic wave-absorbing surface modified particles (Fig. 4), such particles provide for the preparation of liposome compositions which offer a method for the treatment of cancer, as well as infectious diseases.

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PREPARATION OF CONTROLLED SIZE INORGANIC PARTICLES FOR USE IN SEPARATIONS, AS MAGNETIC MOLECULAR SWITCHES, AND AS INORGANIC LIPOSOMES FOR MEDICAL APPLICATIONS

Field of the Invention

This invention relates to a method for producing inorganic oxides of substantially uniform particle size distribution, coating said particles with various functional moieties, and clustering said moieties together via controllably degradable chemical, complex, or ionic bonds. More particularly, this invention relates to a method of producing magnetic inorganic oxide particles of substantially uniform size, or organic coated particle beads, linking the particle or particle bead together to form a large aggregate cluster with different chemical, physical, or magnetic properties than the unit particle or bead, and controllably and predictably revising the cluster back to unit bead or particle size and vice versa.

The substantially uniform size inorganic oxides also allow for the preparation of novel inorganic core liposome compositions for *in vivo* and *in vitro* medical applications.

Background of the Invention

Separations of all types are routinely done by the exploitation of physical and chemical differences in the various species to be separated. Size exclusion, boiling point, and chemical affinity are techniques that have been used for separations of particles, chemical species, and biological moieties for hundreds of years. More recently, the use of magnetism has been used as a tool for separation of various species material from one another. By the early 1960's, the first stable magnetic fluid colloid had been described. Later research led to the development of a separations device based on magnetic density gradients in magnetic fluid columns. By 1979, magnetic particles coated with appropriate functional

chemical groups for affinity chromatography separations were reported. The first commercial application of magnetic separations was described by Chagnon et al in U.S. Patent No. 4,628,037. The Chagnon patent describes the use of amine terminated silane coupled magnetic particles for immunodiagnostic applications. The materials described in the Chagnon et al patent are now used commercially in medical diagnostic kits.

Magnetic separations have not been exclusively applied to in vitro applications. The use of magnetic separations for in vivo applications is becoming increasingly more accepted and important as a therapeutic and diagnostic tool. By the early 1980's, published reports described the magnetic targeting and isolation of chemotherapeutic drugs into rat-tail sarcoma. Widder (U.S. Patent Nos. 4,849,210; 4,247,406; and 4,230,685) describe the use of magnetic albumin spheres for ultrasound contrast media and magnetic drug targeting. Schroeder (U.S. Patent No. 4,501,726) reports a method of preparing magnetic starch beads for use in MRI imaging for the separation of T_1/T_2 relaxation signals.

In all of this previous work, the use of magnetic separations has been done on magnetic particles of varying particle size distribution. The magnetic particle is coated with an organic compound, and used either as a signal (e.g., MRI), targeting agent (e.g. in drug delivery) or for separation in a magnetic field (e.g. in vitro separations). However, an advantage in enhanced separations, for example, could be achieved if the magnetic particle could alter its size, shape or magnetic properties while in use in a controlled fashion.

Various methods have been reported for preparing inorganic or inorganic oxide particles of some degree of particle size control:

U.S. Patent 5,071,076 describes a method for producing magnetic microparticles from metallocenes. The method

involves combining an aqueous slurry of the metallocene and an aqueous slurry of a metal hydroxide and milling the slurries together.

U.S. Patent 4,987,012 describes a process for preparing spherical particles of hydroxide having a particle diameter from 0.1 to 10.mu.m by adding a corresponding metal alkoxide to a dispersion of a water-alcohol system having dispersed therein a metal oxide or hydroxide as a seed, under alkaline conditions and allowing a decomposition product from said metal alkoxide to attach onto said seed to effect particle growth of the seed. The improvement reported comprises maintaining said dispersion at a substantially constant pH within the range between 10 and 13 during the addition of the metal alkoxide to said dispersion and the subsequent particle growth of the seed, thereby to prepare mono-dispersed particles substantially free from particle aggregation having a sharp particle size distribution of a standard deviation of not greater than 0.5.

U.S. Patent 4,985,273 describes a method of producing fine inorganic particles. The method comprises the steps of reacting an inorganic fine particle on the entire surface thereof with a silane type surface active agent containing a straight hydrocarbon chain and a functional group to form a monomolecular film on the entire surface of said inorganic fine particle, thereafter making the inorganic fine particles covered with the monomolecular film in a predetermined density on a substrate, and thereafter subjecting the monomolecular film to physical or chemical treatment to allow the functional groups to be chemically bonded to each other.

U.S. Patent 4,945,049, reports on a method for preparing magnetic powder comprising homogeneous and fine particles using an alkali-producing enzyme. Particles having a particle size ranging from 50 to 500 nm's were reported.

U.S. Patent 4,702,775 describes the control of particle size in the preparation of magnetite pigments. The mean particle size was brought to a value within the range of 0.06 to 0.5 .mu.m by means of a residence stage between the precipitation stage and the oxidation stage.

Various other disclosures describe the preparation of microporous membranes, primarily for a filtration purpose, which limit the passage of selected size molecules within a particular liquid medium. For example, U.S. Patent 4,943,374 concerns the use of a microporous membrane constructed of a polyether sulfone and hydrophilization agent having a pore size which is within the range of 0.1 and 1.2 microns for the filtration of beer. U.S. Patent 4,954,381 describes the preparation of porous substrates having well defined morphology. U.S. Patent 4,964,992 describes a membrane filter having predetermined controlled porosity and to the method for making such a membrane filter. U.S. Patent 5,057,226 describes a method of removing a constituent of a biological fluid including a blood component, said method including flowing the biological fluid past one side of a first semipermeable membrane, flowing solution containing a first precipitation agent past a second side of the membrane so as to cause transfer of the precipitation agent through the membrane to the biological fluid so as to improve precipitation characteristics of the fluid; and precipitating the constituent.

What emerges from the above, therefore, is the lack of a convenient method to control inorganic oxide particle size, such that particle size control can then be further utilized to manufacture novel aggregate particle clusters with unique chemical or physical-chemical properties.

Accordingly, it is an object of this invention to provide a method for producing inorganic oxides of substantially uniform particle size, coating said particles with various functional moieties, and clustering

said moieties together via controllably degradable chemical, complex or ionic bonds.

It is also an object of this invention to provide a method of producing magnetic particle or organic coated particle beads, linking said particle or particle beads together to form a large aggregate cluster with different chemical, physical, or magnetic properties than the unit particle or bead from which it is derived, and controllably and predictably revising the cluster back to unit bead or particle, and vice versa.

It is also a further object of this invention to provide a method of producing unit magnetic crystals of small, substantially uniform particle size for use in preparing magnetic-molecular switches and apply such to several in vitro and in vivo medical and biological applications.

Nomenclature

The term "magnetic crystal" is defined as a particle 10A to 10,000 A in diameter comprised of iron oxide, iron metal, cobalt metal, nickel metal, magnetic ferrites, magnetic alloys, or mixed lattice magnetic metals or metal oxides. The term "magnetic bead" is defined as a magnetic crystal or population of crystals coated by an organic moiety or polymer or inorganic moiety or polymer to form a bead of 10A to 500,000 A in diameter. The term "magneto-molecular switch" is defined as a cluster of magnetic crystals or beads formed by the attachment of organic moieties to the surface of the crystal or beads that link the beads or crystals together via controllably degradable chemical, complex, or ionic bonds.

As used herein the term:

"Polyalkylether" refers to polyethyleneglycol and related homopolymers, such as polymethylethyleneglycol, polyhydroxypropyleneglycol, polypropyleneglycol, poly-methylpropyleneglycol, and polyhydroxypropyleneoxide, and to heteropolymers of small alkoxy monomers, such as

polyethylene/polypropyleneglycol, such polymers having a molecular weight of at least about 120 daltons, and up to about 20,000 daltons.

"Amphipathic organic compound" refers to any organic compound containing both a hydrophobic and hydrophilic moiety.

"Amphipathic vesicle forming lipid" refers to any lipid having a hydrophobic unit and hydrophilic unit, the hydrophobic group typically including two acyl hydrocarbon chains, the hydrophilic group containing a reactive chemical group such as amine, acid, ester, aldehyde, or alcohol group by which the lipid can be derivatized, e.g. to a polyalkylether.

Summary of the Invention

This invention provides a method for preparing novel precipitated inorganic oxide crystal particles of substantially uniform particle size distribution. The method comprises contacting aqueous solutions of an inorganic salt and an inorganic base across a porous membrane wherein the membrane contains a plurality of pores which allows for precipitation of substantially mono-dispersed inorganic oxide particles on one side of the membrane and precipitation of a salt of the corresponding base on a second side of the membrane.

When the inorganic oxide crystal particles produced according to this method is an iron oxide particle of reduced particle size (e.g. Fe_3O_4), which are non-magnetic, they can be aggregated into one embodiment of the magneto-molecular switch which comprises attachment of organic moieties to the surface of the crystals that link the crystal together to from controllably degradable chemical, complex or ionic bonds. It has also been found that aggregate clusters of crystals can be prepared by air or inert gas drying of the crystal particles along with several different solution encapsulation techniques.

In a further embodiment of the magneto-molecular

switch, the individual crystal particles or population of crystals so produced are coated by polymer encapsulation, adsorbtion of monomer followed by crosslinking, or by applying organo-metallic polymer coatings which are covalently bonded or adsorbed onto said particles, to form a non-reversibly coated bead of 10A to 500,000 A in diameter. Accordingly, the beads themselves can be aggregated into controllably degradable bead clusters by the organic moieties that may be present on the beads, or by further attachment of organic moieties to the bead surface, which in either case allow the beads to link together to form controllably degradable chemical, complex, or ionic bonds.

The present invention relates in one aspect to a coated magnetically responsive particle comprising a magnetic core particle comprising a magnetically responsive metal, metal alloy or metal oxide and an organo-metallic polymer coating covalently bonded to said particle wherein the bonding does not depend on the presence of hydroxy functionality on the surface of said particle, and wherein the organo-metallic polymer coating is capable of binding at least one type of bioaffinity adsorbent. In addition to covalent bonding, the organo-metallic polymer can be adsorbed. The coated magnetically responsive particles have utility for either the separation or directed movement of biological molecules from a surrounding medium.

The organo-metallic polymer is formed from an organo-metallic monomer, which is applied to the metal particle, and thermally cross-linked in situ to form an adsorbed or a covalently bound polymer coating. Organo-titanium polymers are preferred, however, organo-metallic polymers formed from coordinate complexes of other transition metals, such as zirconium (Zr), hafnium (Hf), vanadium (V), tantalum (Ta) and niobium (Nb) or post-transition metals, such as tin (Sn) and antimony (Sb), can be used.

A wide variety of bioaffinity adsorbents can be covalently bonded to the organo-metallic polymer coating through selected coupling chemistries.

More particularly, the invention relates to methods for the preparation of magnetically responsive particles comprising a metal, metal alloy or metal oxide core and an organo-metallic coating having an aliphatic moiety and an organic functionality to which a variety of organic and/or biological molecules can be coupled. The particles, coupled or uncoupled, can be dispersed in aqueous media forming a colloidal dispersion which is stable, that is, the particles resist rapid gravitational settling. The particles can be reclaimed from the media by applying a magnetic field.

Preferably, the particles are superparamagnetic; that is, they exhibit no reminent magnetization after removal of a magnetic field which allows the particles to be redispersed without magnetic aggregate formation.

The organo-metallic coated magnetically responsive particles of the invention may be coupled through the organic functionality to biological or organic molecules with affinity for, or the ability to adsorb, or which interact with, certain other biological or organic molecules. Particles so coupled may be used in a variety of in vitro or in vivo systems involving separations steps or the directed movement of coupled molecules to particular sites, including immunological assays, other biological assays, biochemical or enzymatic reactions, affinity chromatographic purification, cell sorting and diagnostic and therapeutic uses.

In connection with the above, and in a further aspect of the present invention, a method of measuring analytes in a sample is disclosed comprising the steps of: (a) contacting a sample containing an unknown concentration of the analyte with a known amount of a labeled analyte in the presence of magnetic particles comprising: (1) a

magnetic core particle comprising a magnetically responsive metal, metal alloy or metal oxide; and (2) an organo-metallic polymer coating covalently bonded to said particle wherein the bonding does not depend on the presence of hydroxy functionality on the surface of said particles, and wherein said organo-metallic coating has a bioaffinity adsorbent covalently coupled thereto, said bioaffinity adsorbent is capable of binding to or interacting with both the unlabeled and the labeled analyte; (b) maintaining the mixture in step (a) under conditions sufficient for said binding or interaction to occur; (c) magnetically separating the magnetic particles; and (d) measuring the amount of label associated with the magnetic particles and determining the concentration of analyte in solution.

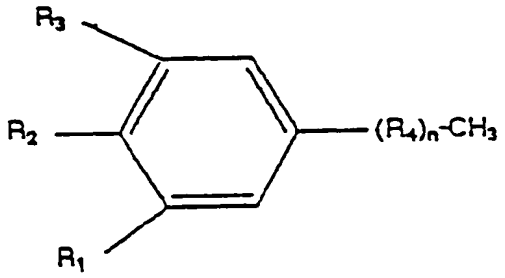
The present organo-metallic coated magnetic particles provide superior composition, size, surface area, coupling versatility, settling properties, and magnetic behavior for use in biological separations. The magnetic particles of this invention are suitable for many of the assays, enzyme immobilization, cell sorting and affinity chromatography procedures reported in the literature and, in fact overcome many of the problems associated with particle settling and reuse experienced in the past with such procedures.

It has now been found that the inorganic oxides of substantially uniform particle size can be used to prepare a liposome composition comprising a substantially uniform size inorganic core coated with an amphipathic organic compound and further coated with a second amphipathic vesicle forming lipid. The inorganic core is again prepared by contacting aqueous solutions of an inorganic salt and an inorganic base across a porous membrane wherein the membrane contains a plurality of pores which allows for precipitation of substantially monodispersed size inorganic oxide particles on one side of the membrane

and precipitation of a salt of the corresponding base on a second side of the membrane. Inorganic cores are also prepared by the reaction of metallocenes with aqueous metal hydroxide slurries followed by milling to uniform particle size. The class of inorganic cores include Fe_3O_4, Fe_2O_3, Al_2O_3, TiO_2, ZnO, FeO, and Fe.

The amphipathic vesicle forming lipid is preferably a lipid having two hydrocarbon chains, including acyl chains, and a polar head group. Included in this class are the phospholipids, such a phosphatidylcholine (PC), phosphatidic acid (PA), phosphatidylinositol (PI), sphingomyelin (SM), and the glycolipids, such as cerebroside and gangliosides.

The amphipathic vesicle forming lipid can also be a novel synthetic phenyl lipid compound having the structural formula:



wherein two of R_1, R_2 and R_3 represent a saturated or unsaturated straight-chain or branched chain alkyl or acyl group, the other being hydrogen, therein providing at least two hydrocarbon chains attached to the phenyl moiety, wherein the two hydrocarbon chains are typically between about 14-22 carbon atoms in length, and have varying degrees of unsaturation. R_4 represents the repeating unit of either a poly(alkylene oxide) polymer, preferably ethylene, propylene and mixtures thereof, or the repeating unit of poly(vinyl alcohol). The number of alkylene oxide or vinyl alcohol groups in the polymer, designated as n, may vary from 0 to about 200 or more.

In a further aspect, the invention includes an inorganic core liposome composition for administering

drugs via the bloodstream, comprising a substantially uniform size inorganic core coated with an amphipathic organic compound and further coated with 1-20 mole percent of an amphipathic vesicle-forming lipid derivatized with a hydrophilic polymer, and containing the compound in liposome-entrapped form.

It has now also been found that liposome compositions can be prepared to comprise a wave absorbing magnetic core coated with an amphipathic organic compound and further coated with a second amphipathic vesicle forming lipid. In a preferred embodiment, the wave absorbing magnetic core particles comprise ferrite or mixed ferrite materials, preferably of a uniform, controllable size and narrow size distribution, wherein the primary component, the oxide, is of the formula $M_2(+3)M(+2)O_4$, wherein M(+3) is Al, Cr or Fe, and M(+2) is Fe, Ni, Co, Zn, Zr, Sr, Ca, Ba, Mg, Ga, Gd, Mn or Cd. In a further aspect, the oxides can be advantageously mixed with LiO, MaO and KO, or with Fe_2O_3 and Fe_3O_4.

The preparation of substantially uniform size oxides, 1 to 50,000 nm in diameter, is achieved by conversion of hydrous oxide gels, in a multi-step process, wherein alkali is added to individual M(+3) and M(+2) aqueous solutions, which separately precipitate the corresponding metal hydroxide. The two precipitates are then coarsely mixed to provide micron size amphorous gel particles, or the gels can be finally mixed by ball milling, for example, to a particle size of about 100 A in diameter. These particles are then heated to effect dehydration, in the presence of oxygen or air, wherein the dehydration temperature, time of dehydration, and concentration of oxygen or air operate to control the particle size of the oxide crystals therein produced.

In a further aspect, the invention includes a process for the treatment of cancer cells by application of external electromagnetic energy capable of the generation

of heat in intracellular particles to induce selective thermal death of cancer cells comprising intravenously injecting into the patient a wave absorbing magnetic core particle coated with an amphipathic organic compound and further coated with a second amphipathic vesicle forming lipid, absorbing said coated wave absorbing magnetic core particle intracellulary into the cancer cells, subjecting the patient to an alternating electromagnetic field to inductively heat the magnetic core particle and thereby the cancer cells, and continuing the inductive heating of said magnetic core particle to attain an increase in intracellular temperature to selectively kill the cancer cells.

Brief Description of the Figures

Fig. 1 is a drawing of a precipitation chamber used in accordance with the present invention.

Fig. 2 illustrates the general liposome composition comprising a substantially uniform size inorganic core coated with an amphipathic organic compound and further coated with an amphipathic vesicle forming lipid.

Fig. 3 is a reaction scheme for preparing a phenyl lipid derivatized with polyethyleneglycol.

Fig. 4 illustrates the general liposome composition comprising a wave absorbing magnetic core particle coated with an amphipathic organic compound and further coated with an amphipathic vesicle forming lipid.

Detailed Description of The Invention

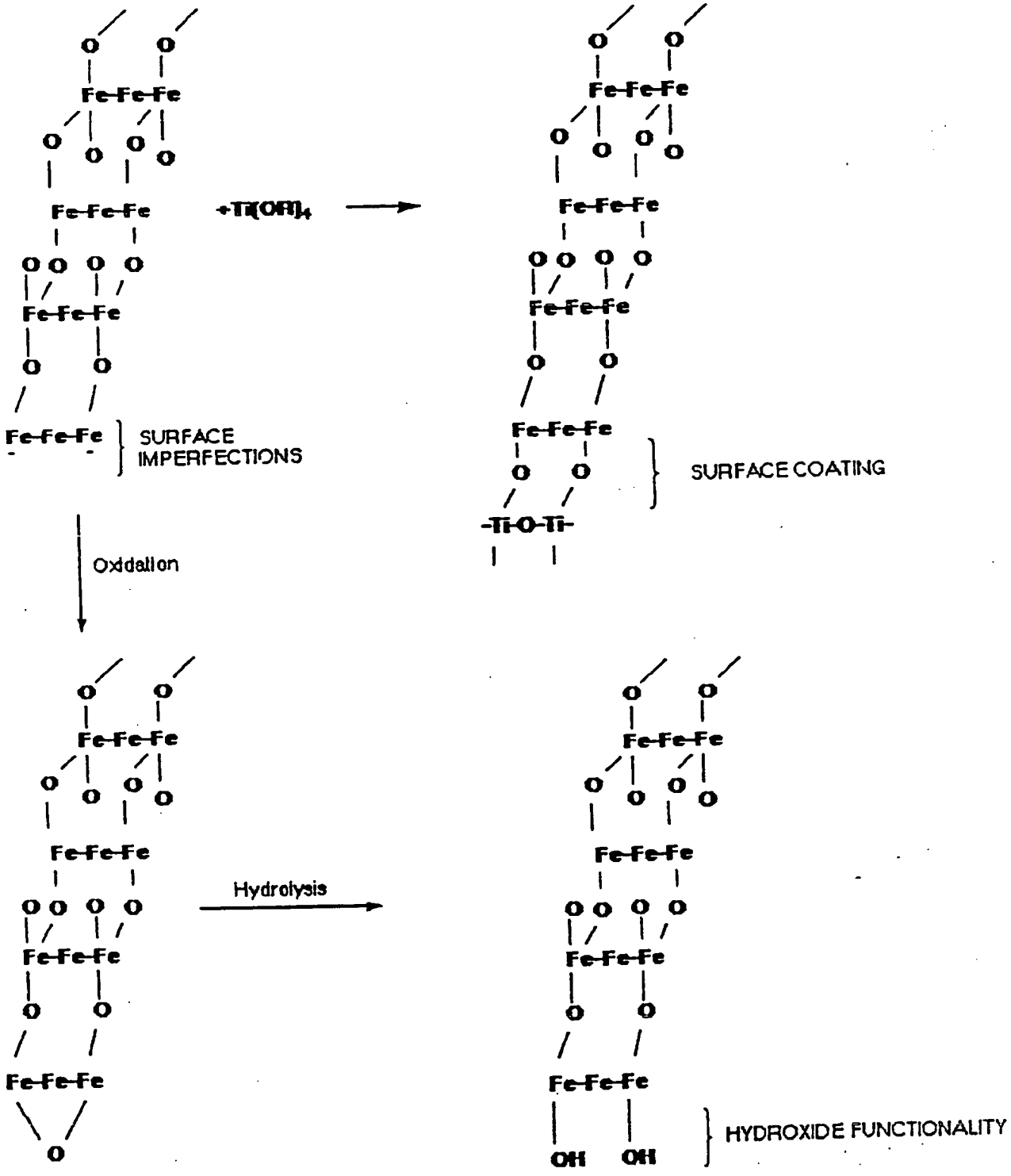
The magnetically responsive particles of this invention overcome problems associated with the size, surface area, gravitational settling rate and magnetic character of previously developed magnetic particles. Gravitational settling times in excess of about 24 hours can be achieved with the present magnetic particles. The gravitational settling time is defined to be the time for the turbidity of a dispersion of particles to fall by fifty percent in the absence of a magnetic field gradient.

The present magnetic particles comprise a core of a magnetically responsive metal, metal alloy or metal oxide, coated with organo-metallic polymer, which is capable of binding reactive groups or agents, for example, chemically reactive groups, biologically reactive groups or bioaffinity agents. The organo-metallic polymer is adsorbed onto or covalently bound to the magnetic particle. The term "magnetically responsive particle" or "magnetic particle" is defined as any particle dispersible or suspendible in aqueous media without significant gravitational settling, and separable from suspension by application of a magnetic field.

The term "magnetic core" is defined as a crystal or group (or cluster) of crystals of a transition metal, alloy or magnetic metal oxide having ferrospinel structure and comprising trivalent and divalent cations of the same or different transition metals or magnetic metal crystal group. Metals, alloys and oxides which are useful as magnetic core material in the present invention include the metals, alloys and oxides based on metals which appear in the Periodic Table in Groups 4a and b, 5a and b, 6a and 7a. These include, for example, divalent transition metals, such as iron, magnesium, manganese, cobalt, nickel, zinc and copper, alloys of these metals such as iron alloys or oxides (e.g., iron magnesium oxide, iron manganese oxide, iron cobalt oxide, iron nickel oxide, iron zinc oxide and iron copper oxide), cobalt ferrite, samarium cobalt, barium ferrite, and aluminum-nickel-cobalt and metal oxides including magnetite (Fe_3O_4), hematite (Fe_2O_3) and chromium dioxide (CrO_2). By way of illustration, a magnetic core may be

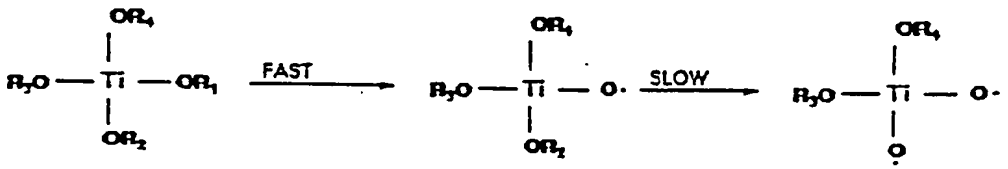
comprised of a cluster of superparamagnetic crystals or iron oxide, or a cluster of superparamagnetic or ferromagnetic crystals of irons or oxide, or may consist of a single superparamagnetic or ferromagntic crystal of an iron oxide or metal alloy.

It has now been found that the Fe_3O_4 affords a crystal lattice which contains primarily trivalent iron (Fe+3) at or near the surface of the crystal. These "surface trivalent" elements of the lattice contain imperfections which make them available for direct covalent attachment of the organometallic compounds of the formula $Ti(OR)_4$ according to the following general equation:


+Ti(OH)4
SURFACE IMPERFECTIONS
SURFACE COATING
Oxidation
Hydrolysis
HYDROXIDE FUNCTIONALITY

It should be noted that the imperfections of the surface trivalent iron are somewhat short-lived, and if organo-metallic coating is delayed, oxidation and hydrolysis can occur causing the development of surface hydroxyls which preclude direct covalent attachment of the organo-metallic moiety. For example, freshly made Fe_3O_4 will spontaneously react; Fe_3O_4 material after 24 hours reacts but requires about 1 hour of dwell time; after 48 hours the coupling reaction takes place very slowly and is generally incomplete.

Organo-metallic compounds are preferably of the formula $Ti(OR)_4$ wherein R is an alkyl group and the dissociation to the reactive component follows the following general reaction criterion:



Accordingly, R_1, R_2, R_3 and R_4 are selected so that rapid dissociation of the first radical (R_1) is fast, and dissociation of subsequent radicals (R_2-R_4) is slow. It has been found that when the radicals R_1-R_4 are collectively alkyl type, the dissociation is linear with respect to the length of the chain (the shorter the chain, the faster the dissociation). Therefore it is possible to shift the reactivity of such organo-metallic compounds by simply replacing shorter alkyl substituents with longer alkyl substitution. It has also been found that when R is an aryl moiety, dissociation is relatively slow. Other

moieties (e.g. esters, ketones) have been found to provide intermediate dissociation constants.

The present particles are preferably between about 0.003 and about 1.5 microns in diameter, and have a surface area of from about 50 to 150 meters/gm, which provides a high capacity for coupling of a bioaffinity adsorbent, chemical or biochemical reactive group. Magnetic particles of this size range overcome the rapid settling problems of larger particles, but obviate the need for large magnets to generate the magnetic fields and magnetic field gradients required to separate smaller particles. For example, magnets used to effect separations of the magnetic particles of this invention need only generate magnetic fields between about 100 and about 1000 Oersteds. Such fields can be obtained with permanent magnets which are smaller than the container which holds the dispersion of magnetic particles and, thus, are suitable for benchtop use.

Particles with superparamagnetic behavior are preferred since superparamagnetic particles do not exhibit the magnetic aggregation associated with ferromagnetic particles and permit redispersion and reuse. The term "superparamagnetism" is defined as that magnetic behavior exhibited by iron, cobalt, nickel or other metal alloys or metal oxides having a crystal size of less than about 300A, which behavior is characterized by

responsiveness to a magnetic field without reminant magnetization.

Ferromagnetic particles may be useful in certain applications of the invention. The term "ferromagnetism" is defined as that magnetic behavior exhibited by iron, iron alloys or iron oxides with a crystal size greater than about 500A, which behavior is characterized by responsiveness to a magnetic field with a reminant magnetization of greater than about 10 gauss upon removal of the magnetic field.

The particles or crystals are then coated with an organo-metallic monomer material capable of adsorptive or covalently bonding to the magnetic particles. Organo-metallic monomers useful for the present coated particles are organic coordinate complexes of selected transition and/or post transition metals which are capable of forming a stable coordination compound, and organic ligands, which can be adsorbed onto or covalently bound to the magnetic particle and, crosslinked in situ on the particle surface, thereby forming the organo-metallic polymer coating. The organo-metallic monomer must be able to be functionalized or derivatized in a manner that allows the polymer formed therefrom to form covalent bonds with bioaffinity or chemical affinity adsorbents. For this purpose, the organo-metallic polymer is post-functionalized or derivitized with an aliphatic "spacer arm" which is terminated with an organic functional group capable of coupling with bioaffinity adsorbents. The "spacer arm" is an aliphatic hydrocarbon having from about 2 to about 60 atoms, e.g., carbon, nitrogen and/or oxygen atoms. The purpose of the spacer arm is to

provide a non-reactive linker (or spacer) between the organic group which reacts with the chemical group, biochemical group or bioaffinity adsorbent and the polymer chain, and to impart an appropriate degree of hydrophilic/hydrophobic balance to the surface of the coated particle. The organic group is generally a reactive group such as an amine (NH_2), carboxyl group (COOH), cyanate (CN), phosphate (PO_3H), sulfate (SO_3H), thiol (SH), hydroxyl (OH) group, vinyl (C=C), nitrate (NO_2), aldehyde, epoxide, succinamide or anhydride group coupled to an aliphatic or aromatic moiety.

Particularly useful organo-metallic compounds are coordinate complexes formed from selected transition metals (e.g., Ti, Zr, Hf, V, Zn, Cd, Mn, Te, Re, Ta, Nb) and/or post-transition metals (e.g., Sn, Sb, Al, Ga, In, Ge). Organo-titanium compounds are particularly preferred. Organo-titanium compounds which are useful including, for example, titanium-tetra-isopropoxide, amino-hexyl-titanium-tri-isopropoxide, amino-propyl-titanium-tri-isopropoxide and carboxyl-hexyl-titanium-tri-isopropoxide. In one embodiment of the present invention, amino-hexyl-titanium-tri-isoproxide is coated onto the magnetic particle of choice, and thermally crosslinked to form an organo-titanium polymer coating, having an aliphatic spacer arm (the hexyl moiety) and organic functional group (the amine group).

The coated particle is post-functionalized, if necessary, in a manner that allows the organo-metallic polymer to form covalent bonds with bioaffinity or chemical affinity adsorbents. In one embodiment of the present method, an organo-titanium polymer, such as

titanium-tetra-isopropoxide which lacks the spacer arm and organic functional group, is coated onto the magnetic particle of choice and partly crosslinked at about 40°C for a period of time sufficient to allow the organotitanium polymer to become adsorbed onto the particle surface. The organotitanium coated magnetic particle is then activated by reaction with an agent such as 1-hydroxy-6-amino hexane, to form the amino-hexyl-titanium-tri-isopropoxide. The coating is then crosslinked at elevated temperatures to form an organotitanium polymer coating having an aliphatic spacer arm and an organic functionality (i.e., the amine group). The functionalized particle can then be reacted or coupled, with the bioaffinity adsorbent of choice.

The magnetic core particles are prepared according to the following general procedure: metal salts are precipitated in a base to form fine magnetic metal oxide crystals. The crystals are redispersed, then washed in water and in an electrolyte. Magnetic separation can be used to collect the crystals between washes if the crystals are superparamagnetic.

In one embodiment of the present invention, superparamagnetic iron oxide particles are made by precipitation of divalent (Fe^{2+}) and trivalent (Fe^{3+}) iron salts, for example, ferrous ammonium sulfate, $Fe_2(NH_2)(SO_4)$ and ferric sulfate, $Fe_2(SO_4)_3$, in aqueous base. The ratio of Fe^{2+} and Fe^{3+} and counterion can be varied without substantial changes in the final product by increasing the amount of Fe^{2+} while maintaining a constant molar amount of iron. Counterions including nitrate, sulfate, chloride or hydroxide are useful in the method. A

Fe^{2+}/Fe^{3+} ratio of about 2:1 to about 4:1 is useful in the present invention; a ratio of about 2:1 $Fe^{2+}:Fe^{3+}$ is particularly useful. An Fe^{2+}/Fe^{3+} ratio of 1:1 produces magnetic particles of slightly inferior quality to those resulting from the higher Fe^{2+}/Fe^{3+} ratios, the particle size is more heterogeneous than that resulting from Fe^{3+}/Fe^{2+} of 2:1 or 4:1.

In this embodiment, aqueous solutions of the iron salts are mixed in a base, such as ammonium, sodium or potassium hydroxide, which results in the formation of a crystalline precipitate of superparamagnetic iron oxide. The precipitate is washed repeatedly with water by magnetically separating and redispersing it until a neutral pH is reached. The precipitate is then washed with about five equal portions of a water miscible solvent, such as acetone, methanol or ethanol that has been dried over molecular sieves to remove all of the water.

The repeated use of magnetic fields to separate the iron oxide from suspension during the washing steps is facilitated by the superparamagnetic properties of the crystals. Regardless of how many times the particles are subjected to magnetic fields, they never become magnetically agglomerated and consequently, can be redispersed by mild agitation. Ferromagnetic particles cannot be prepared by this washing procedure as they tend to magnetically aggregate after exposure to magnetic fields and cannot be homogeneously redispersed.

Other divalent transition metal salts such as magnesium, manganese, cobalt, nickel, zinc and copper salts may be substituted for iron salts in the

precipitation or milling procedure to yield magnetic metals or metal oxides. For example, the substitution of divalent cobalt chloride ($CoCl_2$) for $FeCl_2$ in the above procedure produced ferromagnetic metal oxide particles. Ferromagnetic metal oxide particles such as those produced with $CoCl_2$ can be washed in the absence of magnetic fields by employing conventional techniques of centrifugation or filtration between washings to avoid magnetizing the particles. As long as the resulting ferromagnetic metal oxides are of sufficiently small diameter to remain dispersed in aqueous media, they can also be coated with the organo-metallic polymer and coupled to bioaffinity adsorbents for use in systems requiring a single magnetic separation, e.g., certain radioimmunoassays. Ferromagnetism limits particle usefulness in those applications requiring redispersion or reuse.

In another embodiment of the present invention, the magnetic core particles can be made by precipitating metal powders and reducing the particle size by milling the resulting precipitate, for example, in a ball mill. In this process, the metal powder is precipitated from an aqueous solution of, for example, Fe^{+2} or Fe^{+3} salt with sodium borohydride. For example, an aqueous solution of ferrous chloride ($FeCl_2$) is mixed with sodium borohydride ($NaBH_4$) to form a fine iron precipitate. The resulting properties of the metal powder are unaffected by the valance of the counter ion or iron metal salt selected. Complete precipitation occurs spontaneously upon borohydride addition. The magnetic metal powder is then collected by filtration and washed with about five equal

volumes of water to remove all soluble salts, then washed with five equal volumes of dried acetone to remove all residual water. The particle is added as an aqueous slurry in a concentration of about 1-25% to a commercial ball mill filled half way with 1/4" stainless steel balls and milled for 3-30 days. At the completion of the milling period, a superparamagnetic metal slurry is formed and coated and functionalized as the superparamagnetic particles described in the previous section.

In another embodiment of the present invention, the magnetic core particles are made by reacting a metallocene, e.g., particulate ferrocene (dicyclopentadenyliron, $C_{10}H_{10}Fe$) with iron (II) hydroxide. In this embodiment, an aqueous ferrocene (or other metallocene) slurry is prepared, and an aqueous slurry of iron (II) hydroxide is prepared separately. The ferrocene slurry is prepared, for example, by milling a mixture of ferrocene and water in a ball mill. The iron (II) hydroxide slurry can be prepared, for example, by precipitating an aqueous solution of ferrous sulfate with ammonium hydroxide to form ferrous hydroxide. The two slurries are then combined and milled, for example, forming fine magnetite particles. Other metallocene compounds (e.g. nickelocene, cobaltocene) can be mixed with the ferrocene to produce various magnetic ferrite particles. This process is described in detail in U.S. Patent No. 5,071,076, the teachings of which are hereby incorporated by reference.

In one embodiment of the present invention, the coating around the magnetic core particle is amino-propyl-titanium-tri-isopropoxide. The polymerization is performed by redispersing the magnetic particle in an acetone solution, adding the organo-titanium monomer, then crosslinking with heat. The terms "coupled magnetically responsive particle" or "coupled magnetic particle" refer to any magnetic particle to which one or more types of bioaffinity adsorbents are coupled by covalent bonds, which covalent bonds may be amide, ester, ether sulfonamide, disulfide, azo or other suitable organic linkages depending on the functionalities available for bonding on both the coating of the magnetic particle and the bioaffinity adsorbents.

Preferred magnetically responsive particles of the present invention have metal oxide cores composed of clusters of superparamagnetic crystals affording efficient separation of the particles in low magnetic fields (100-1000 Oersteads) while maintaining superparamagnetic properties. Aggregation of particles is controlled during particle synthesis to produce particles which are preferably small enough to avoid substantial gravitational settling over times sufficient to permit dispersions of the particles to be used in an intended biological assay or other application. The advantage of having superparamagnetic cores in magnetically responsive particles is that such particles can be repeatedly exposed to magnetic fields. Superparamagnetic particles do not exhibit reminent magnetization and have no coercive strength, and, therefore, do not magnetically aggregate, thus, the particles can be redispersed and

reused. Even after coating, preferred particles of the invention having cores made up of clusters of crystals exhibit a remarkably high surface area per unit weight and a generally corresponding high coupling capacity, which indicates that such particles have an open or porous structure.

The bioaffinity adsorbents can be covalently bonded to the organo-metallic coated magnetic particles of this invention by conventional coupling chemistries. Several coupling reactions can be performed. For example:

(a) If the ligand to be coupled contains an amino group, it can be coupled directly to the activated organo-metallic polymer. If a different functionality is desired, it can be introduced, for example, by adding a spacer arm containing the functionality by sequential reaction of the organo-metallic polymer (e.g., titanium-tetra-isopropoxide) with any omega-functional higher molecular weight alcohol. The amino group on the ligand can then be coupled to the free functional group on the spacer arm; or

(b) If the ligand contains an aldehyde group instead of an amino group, it can be coupled directly to the free amino group of an amino alkane (that is, an alkane spacer arm having an amino functionality) on the coated magnetic particle.

The term "bioaffinity adsorbent" is defined as any biological or other organic molecule capable of specific or nonspecific binding or interaction with another biological molecule, which binding or interaction may be referred to as "ligand/ligate" binding or interaction and is exemplified by, but n t limited to, antibody/antigen,

antibody/hapten, enzyme/substrate, carrier protein/substrate, lectin/carbohydrate, receptor/hormone, receptor/effector or repressor/inducer bindings or interactions.

The coupled organo-metallic coated magnetic particles of the present invention can be used in immunoassays or other binding assays for the measurement of analytes in solution. The term "immunoassay" is defined as any method for measuring the concentration or amount of an analyte in a solution based on the immunological binding or interaction of a polyclonal or monoclonal antibody and an antigen, which method (a) requires a separation of bound from unbound analyte; (b) employs a radioisotopic, fluorometric, enzymatic, chemiluminescent or other label as the means for measuring the bound and/or unbound analyte; and (c) may be described as "competitive" if the amount of bound measurable label is generally inversely proportional to the amount of analyte originally in solution or "non-competitive" if the amount of bound measurable label is generally directly proportional to the amount of analyte originally in the solution. Label may be in the antigen, the antibody, or in double antibody methods, the second antibody. Immunoassays are exemplified by, but are not limited to, radioimmunoassays (RIA), immunoradiometric assays (IRMA), fluoroimmunoassays (FIA), enzyme immunoassays (EIA), and sandwich method immunoassays. The analyte or the bioaffinity adsorbent can include, for example, antibodies, antigens, haptens, enzymes, apoenzymes, enzymatic substrates, enzymatic inhibitors, cofactors, nucleic acids, binding proteins, carrier proteins, compounds bound by binding proteins, compounds bound by carrier

proteins, lectins, monosaccharides, polysaccharides, hormones, receptors, repressors and inducers.

Such assays are preferably carried out by mixing a sample containing an unknown concentration of analyte with a known amount of labeled analyte in the presence of magnetic particles coupled to a bioaffinity adsorbent capable of binding to, or interacting with, both unlabeled and labeled analyte, allowing the binding or interaction to occur, magnetically separating the particles, measuring the amount of label associated with the magnetic particles and comparing the amount of label to a standard curve to determine the concentration of analyte in the sample.

The term "binding assay" or "non-immune assay" is defined as any method for measuring the concentration or amount of an analyte in solution based on the specific or nonspecific binding or interaction, other than antibody/antigen binding or interaction, or a bioaffinity adsorbent and another biological or organic molecule, which method (a) requires a separation of bound from unbound analyte; (b) employs a radioisotopic, fluorometric, enzymatic, chemiluminescent or other label as as the means for measuring the bound and/or unbound analyte; and (c) may be described as "competitive" if the amount of bound measurable label is generally inversely proportional to the amount of analyte originally in solution or "non-competitive" if the amount of bound measurable label is generally originally in solution.

The magnetic organo-metallic-coated particles of this invention are useful in immobilized enzyme systems, particularly where enzyme recycling is desired. The term

"immobilized enzyme system" is defined as any enzymatically catalyzed biochemical conversion or synthesis or degradation wherein the enzyme molecule or active site thereof is not freely soluble but is adsorptively or covalently bound to a solid phase support, which support is suspended in or contacted with the surrounding medium and which may be reclaimed or separated from said method. In this embodiment, enzymatic reactions are carried out by dispersing enzyme-coupled magnetic particles in a reaction mixture containing one or more substrates, under conditions sufficient for the reaction between the enzyme and substrate to occur, magnetically separating the enzyme-magnetic particle from the reaction mixture containing products and unreacted substrates and, if desired, redispersing the particles in fresh substrates thereby reusing the enzyme.

Affinity chromatography separations and cell sorting can be performed using the magnetic particles of this invention. The term "affinity chromatography" is defined as a method for separating, isolating, and/or purifying a selected molecule from its surrounding medium on the basis of its binding or interaction with a bioaffinity adsorbent adsorptively or covalently bound to a solid phase support, which support is suspended in or contacted with the surrounding medium and which may be reclaimed or separated from said medium by dispersing bioaffinity adsorbent coupled magnetic particles in solutions or suspensions containing molecules or cells to be isolated and/or purified, allowing the bioaffinity adsorbent and the desired molecules or cells to interact, magnetically separating the particles from the solutions or suspension

and recovering the isolated molecules or cells from the magnetic particles.

It is further contemplated that the organo-metallic coated magnetic particles of this invention can be used in in vivo systems for the diagnostic localization of cells or tissues recognized by the particular bioaffinity adsorbent coupled to the particle and also for magnetically directed delivery of therapeutic agents coupled to the particles to pathological sites.

Magnetic separation times of less than about ten minutes can be achieved with magnetic particles of the invention by contacting a vessel containing a dispersion of the particles with a pole face of a permanent magnet no larger in volume than the volume of the vessel. Magnetic separation time is defined to be the time for the turbidity of the dispersion to fall by 95 percent.

Furthermore, the use of functionalized organo-metallic polymers as the coating surrounding the metal oxide core of the magnetic particles described herein make possible the coupling of a wide variety of molecules under an equally wide variety of coupling conditions compared to other magnetic particle coatings known in the art with more limited coupling functionalities.

The invention is further illustrated by the following Examples.

EXAMPLES

Example 1: Preparation of Superparamagnetic Magnetite Particles

200 grams (1.58 moles) of ferrous chloride (VWR Scientific) and 325 grams (2.0 moles) of ferric chloride

were dissolved in 3 liters of water. 2000 grams of ammonium hydroxide (VWR Scientific) concentrate were added at a rate of 50 ml/minute under constant agitation, during which time the temperature of the solution was kept between 25 and 40°C. After the addition of the ammonium hydroxide was complete, the magnetic particle (Fe_3O_4) aqueous slurry was allowed to cool to room temperature.

Example 2: Preparation of Amino-Hexyl-Titanium-Tri-Isopropoxide

0.1 moles of titanium-tri-isopropoxide (Tyzor TPT Dupont, Wilmington, DE) and 0.1 moles of 6-amino-1-hexanol were added to a 50 ml beaker and stirred at room temperature for 1 minute to form 0.1 mole of amino-hexyl-titanium-tri-isopropoxide. The reaction mixture was heated to 70°C for 10 minutes to evaporate the isopropyl alcohol formed during the reaction.

The material was cooled to room temperature and used as a monomer in making the tetravalent titanium organo-metallic coating in Example #3.

Example 3: Preparation of Amine Functional Organo-titanate Coated Magnetic Particle

According to the procedure set out in Example 1, 4 moles $FeCl_3$ and 2 moles of $FeCl_2$ were dissolved in 4 L of distilled water and precipitated with 16 moles of ammonium hydroxide. The precipitate was washed 5 times with water and 3 times with acetone. N,N-dimethyl formamide (DMF) was added to the precipitate in the following rati : 10 ml of DMF per gram of Fe_3O_4. The mixture was loaded into a Eiger Mill and milled

continuously for 10 minutes. The mixture was then transferred to a beaker and heated with stirring for 30 minutes at 100°C. The amine functional organo-titanate prepared in Example 2 was immediately added after preparation with constant stirring to the mixture in a ratio of 1 g dry Fe_3O_4 per 3 g of amine functional organo-titanate.

This mixture was then heated with stirring for 20 minutes at 65°C and then passed through the Eiger Mill for two passes. The resulting material was washed five times with water, the coated particles were collected with an external magnetic field of 2000 gauss and the aqueous waste was decanted.

Example 4: Preparation of An Alternating Functional-Non Functional Organo-Titanate Monomer

The procedure described in Example 2 was followed except that the organo-titanate was reacted with a comixture of amino-functional hexanol and hexanol to produce a monomer having reduced amine functionality. Hexanol and 6-amino-1-hexanol in a molar ratio of 6:1 were mixed in a 50 ml beaker for one minute. Tyzor TPT was added to the alcohol mixture in the ratio of 1 mole of alcohol per mole of Tyz or TPT. The reaction mixture was stirred for one minute, heated to 70°C for 10 minutes to evaporate the isopropyl alcohol produced by the reaction and cooled to room temperature. The resulting compound was an organotitanate, 6-amino-hexyl-titanium-tri-isopropoxide having alternating non-functional hexyl groups, that is, hexyl chains lacking the amino group. The weight ratios of 6-amino-1-hexanol:Tyzor TPT:hexanol

were 1:26:9.6. This compound was used as a monomer to make an organo-titanium coating as described in Example 5.

Example 5: Preparation of Amine Functional Organo-titanate Magnetic Particles

The procedure described in Example 3 was followed except that the amine-functional organo-titanate was the material prepared in Example 5. The mixture of magnetic particles and organo-titanate monomer was heated to 95°C for one hour with constant stirring and milled in an Eiger Mill for 4 minutes. The mixture was washed nine times with water. Adipic acid was added in the ratio of 0.5 moles of adipic acid per mole of total particles. One mole of carbodiimide (CDI) was added, and the mixture was mixed for 30 minutes on a ball mill. 1,6 hexane-diamine was added in the ratio of 0.5 moles of 1,6 hexane-diamine per mole of total particles. One mole of CDI was added and the mixture was mixed for 30 minutes. The resulting material was washed five times with water, the particles were collected using an external magnetic field of 2000 gauss and the aqueous waste was decanted.

Example 6: Preparation of Subdomain Magnetite Particles by Reaction of Particulate Ferrocene and Iron(II) Hydroxide

A 100 g of a slurry containing 20% ferrocene (by weight) (dicyclopentadenyliron; Strem Chemical Co., Newburyport, MA) in water was prepared by mixing the ferrocene with the water. The slurry was added to a commercial ball mill. The mill was filled halfway with ¼" stainless steel balls and the slurry was milled for a

period of 2 hours.

A second ferrous hydroxide slurry (iron (II) hydroxide) was made according to the following procedure. An aqueous solution containing 20g of ferrous sulfate (VWR Scientific) was precipitated using 50g of ammonium hydroxide concentrate to form gelatinous ferrous hydroxide. The gel was filtered and the filtrate washed with 5 to 100g volumes of water. The washed gel was then made into a 10% aqueous slurry and milled as previously described for 5 hours.

The ferrocene and hydroxide slurries were mixed, and the mixture was milled for one day to form fine Fe_3O_4 particles. The particles were about 100 A in diameter and were responsive to a magnetic field. These particles can be coated as described in Examples 2-5 above.

Example 7: Preparation of Subdomain Nickel-Ferrite Particles

Subdomain nickel-ferrite particles were prepared according to the procedure set out in Example 6, except that a mixture of 50g a 20% nickelocene slurry (dicyclopentadenylnickel; Strem Chemical Co., Newburyport, MA) and 50g of a 20% ferrocene slurry were used in lieu of the 100g of the ferrocene slurry in Example 6. Magnetically responsive nickel-ferrite particles having a particle size of about 100 A were produced by this method.

Example 8: Preparation Subdomain Cobalt-Ferrite Particles

Subdomain cobalt-ferrite particles were prepared according to the procedure set out in Example 6, except that a mixture of 50g of a 20% (by wt.) cobaltocene

slurry (dicyclopentadenylcobalt; Strem Chemical Co., Newburyport, MA) and 50g of the ferrocene slurry were used in lieu of 100g of the ferrocene slurry in Example 6. Magnetically responsive cobalt-ferrite particles having a particle size of about 100 A were produced by this method.

Example 9: Preparation of Subdomain Metal Particles by Sodium Borohydride Reduction and Size Reduction by Milling

200 gm (1.58 moles) of ferrous chloride was dissolved in 1 liter of water. 500 gm of dry sodium borohydride were added to the solution to form a fine iron powder precipitate. The precipitate was washed with water and collected by filtration. The filtered powder was resuspended in water and re-filtered. The washing procedure was done 4 additional times. On the final suspension, the slurry was adjusted to a concentrate of 20% and milled as described in Example 6 for a period of 75 days to produce particles with a mean diameter of less than 50 A.

Description of the Sub 100A Ferrite Particle

Sub 100A ferrites have been prepared by the co-precipitation of metal(+2) and metal(+3) salts in aqueous solutions with aqueous base across a porous or dialysis membrane. The metal salt solutions are put into a dialysis bag and the bag is sealed. The bag containing the metal salt solution is then immersed in an aqueous solution of base (i.e. ammonium hydroxide) over a period of several minutes to several days, depending on the concentration of the various reactants, and a precipitate of metal oxide forms inside of the dialysis bag. The size of the particles thus prepared is controlled by: concentration of the metal salt solution; concentration of the base solution; pore size of the membrane; temperature of the various solutions; ionic strengths (or ionization constant) of solutions; and the contact times of each solution across the dialysis membrane.

It has further been discovered that metal oxide particles of various controlled size can also be formed by contacting an aqueous solution of metal salts with a dialysis bag filled with aqueous base. In this case, the desired metal oxide product will form outside of the dialysis bag.

In a preferred embodiment, the inorganic base and the inorganic salt solutions are maintained in large volume chambers separated by a porous membrane. Accordingly, large amounts of inorganic oxide of controlled particle size can be produced. As can be seen from Figure 1, a large volume chamber (10) contains a partition (12), a semi-permeable membrane (14), an opening (16), a support (18) for mounting of the membrane, and portals (20) for draining. The metal salt solution is placed on the membrane side of the chamber, such that the metal oxide particles precipitate on that side of the large volume chamber.

It has also been discovered that the size of the cationic moiety on the base side of the membrane controls the size of the precipitated inorganic oxide particle so produced near the surface of the membrane within the inorganic salt solution. Apparently, the speed of dissociation of the inorganic base is believed to be controlled by the size of the cationic moiety; the larger the cationic moiety the slower the dissociation to cationic and anionic component. When the dissociation is relatively slow, a relatively low concentration of anionic moiety is present, providing a relatively low concentration of anion diffusing across the porous membrane and into the inorganic salt solution. Accordingly, the cationic component (of the inorganic salt) exists in large excess, thereby surrounding the slowly diffusing anion, resulting in precipitation of many small-sized inorganic oxide particles.

By contrast, if the cationic moiety of the inorganic base is relatively small, the speed of dissociation is relatively fast, providing a relatively large concentration of anionic moiety diffusing across the porous membrane and into the inorganic salt solution. At the surface of the membrane within the inorganic salt solution the cationic component (of the inorganic salt) once again exists in large excess. Accordingly, while the cationic component surrounds those anionic moieties which have diffused across the membrane, the elevated concentration of diffusing anionic moiety rapidly finds its way to the cationic surface of such a growing particle, so that a further layer of ionic bonding can result, thereby producing larger overall particle size prior to precipitation from solution.

It has been found, for example, that KOH in contact with an aqueous solution of $FeCl_2/FeCl_3$ affords iron oxide particles (Fe_3O_4) that are smaller in size as compared to iron oxide particles produced when LiOH is employed as the

inorganic base. This would comport with the above insofar as the K+ ion is known to be relatively larger than the Li+ ion.

With respect to the foregoing, NH_4OH, KOH, LiOH, NaOH and other hydroxides formed by elements in group Ia of the periodic table serve as suitable inorganic base compounds. Inorganic salt solutions based on mixtures of the type $M^{(+3)}{}_Y/M^{(+2)}{}_Y$ include those wherein Y is selected from the group consisting of Cl, Br, I, SO_4, NO_3 and PO_4. M can be selected from the group consisting of Fe, Co, Ni, Zn, Mn, Mg, Ca, Ba, Sr, Cd, Hg, Al, B, Sc, Ga, V and In. The preferred inorganic salts are those which are readily productive in an aqueous medium of an anion and a cation which can combine with the aforementioned diffusing hydroxide anion to form an inorganic oxide.

Accordingly, inorganic oxide particles of the formula M_3O_4 are prepared wherein M is selected from the group consisting of Fe, Co, Ni, Zn, Mn, Hg, Ca, Ba, Sr, Cd, Hg, Al, B, Sc, Ga, V and In and mixtures thereof. It will also be appreciated that for a given M_3O_4 particle, the metal (M) may often be a combination of different oxidation states of the same metal component. For example, and in the preferred embodiments, Fe_3O_4 particles are prepared and represent a mixed Fe(+2)Fe(+3) oxide of the formula $[Fe(+2)][Fe(+3)]_2O_4$.

With respect to the foregoing, reference is made to the following:

I. <u>The Effect of Alternative Base Counter Ions</u>

The effect of alternative base counter ions on crystal properties such as size, distribution, magnetics, etc. was established as follows: Three experiments were conducted. All experimental conditions were identical except for the type of base. Experiment A utilizes NaOH, B with LiOH and C with KOH. For each experiment: 1. Wash a Spectra/Por® 5 dialysis membrane (cellulose ester based membrane available from Spectrum Medical Industries, Inc.) and

secure over the opening in the dialysis chamber; 2. Fill both sides of the tank with 20 liters of distilled H_2O (at room temperature); 3. Dissolve 12.5g $FeCl_2 \cdot 4H_2O$ in 2 liters of distilled H_2O. Add 20 g $FeCl_3$ and stir until dissolved; 4. Decant all iron solution into the membrane side of chamber; 5. For A dissolve 55g NaOH in 2 liters of distilled H_2O. For B dissolve 55g LiOH in 2 liters of distilled H_2O. For C dissolve 60.6g KOH in 2 liters of distilled H_2O. Decant base solution into opposite side of dialysis chamber. After 70 hours contact time, remove the crystal precipitate solution for evaluation. The results are listed below in Table 1.

Table 1

Sample	Base Ion	Crystal Size(A)	Cluster Size(A)	Magnetics (Gauss)	Iron Conc. (mg/ml)	% Total Solids
A	Na	60-80	300	340	7.0	1
B	Li	120-140	170-250	275	6.44	1
C	K	40	500	187	6.10	1

II. The Effect of Base Concentration

The effect of base concentration on crystal properties such as size, distribution, magnetic response, etc. was established as follows: Two experiments were conducted. All experimental conditions were identical except for base concentration. Experiment A was conducted at 0.5% NaOH. Experiment B was conducted at 0.25% NaOH. For each experiment: 1. Wash a Spectra/Por® 5 dialysis membrane and secure over the opening in the dialysis chamber; 2. Fill both sides of the tank with 20 liters of H_2O (at room temperature); 3. Dissolve 12.5g $FeCl_2 \cdot 4H_2O$ in 2 liters of distilled H_2O. Add 20g $FeCl_3$ and stir until dissolved; 4. Decant all iron solution into the membrane side of chamber; 5. For concentration A: Dissolve 120g NaOH in 2 liters distilled H_2O. Decant into opposite side of tank; For concentration B: Dissolve 55g NaOH in 2 liters distilled H_2O. Decant into opposite side of tank; 6. After 70-80 hours contact time remove iron solution and precipitated crystals for evaluation. The results are listed below in

Table 2.

Table 2

Sample	Crystal Size(A)	Cluster Size(A)	Magnetics Gauss	Iron Conc.	% Total Solids
A	70-80	500	360	7.0mg/ml	1.0
B	60-80	300	340	0.39mg/ml	0.085

It has also been found that the size of the particles may be effected by the following additional variable: the temperature of the solutions; whether the particles formed are removed (including magnetic removal, if the particles are of the appropriate size) from the immediate surface of the membrane; the pore size of the membrane; and whether or not the solutions are stirred. With respect to the pore size, membranes of different molecular-weight cut-offs (MWCO) have been examined. The MWCO represents a limit on the size of the molecule allowed to pass through the pore. MWCO's between 1000 and 500,000 have been investigated. The smaller the MWCO, the smaller the inorganic oxide produced.

Description of Magnetic Clusters

Iron oxide, for example, has been prepared using this technique in sizes of 80A, 50A and 20A, all with a narrow (+/-10%) particle size distribution. A product that agrees with x-ray diffraction patterns for Fe_3O_4 has been prepared in 100, 80, 50 and 20A crystal sizes. The supra 50A particles of Fe_3O_4 have domain magnetization, when measured by a Vibrating Sample Magnetometer (VSM), of 5660 gauss. This result is in agreement with the literature. The sub 50A Fe_3O_4 crystals surprisingly have a very low magnetization. In fact, crystals of 20A Fe_3O_4 have domain magnetization of less than 100 gauss. This low magnetization observed in sub 50A Fe_3O_4 crystals is likely the result of having insufficient mass for spin coupling and the absence of domain wall formation.

Surprisingly, when non-magnetic sub 50A crystals of Fe_3O_4 are clustered together to form aggregates of 250A or

greater, the aggregate particles are strongly magnetic. Aggregate particles of 500A or greater in diameter, when measured by VSM, have domain magnetizations in excess of 4000 gauss.

It has been further discovered that if the aggregates of magnetic crystals are returned to non-aggregated unit sub 50A crystal size, the effect if reversed, that is, the magnetization is returned to nominally 0.

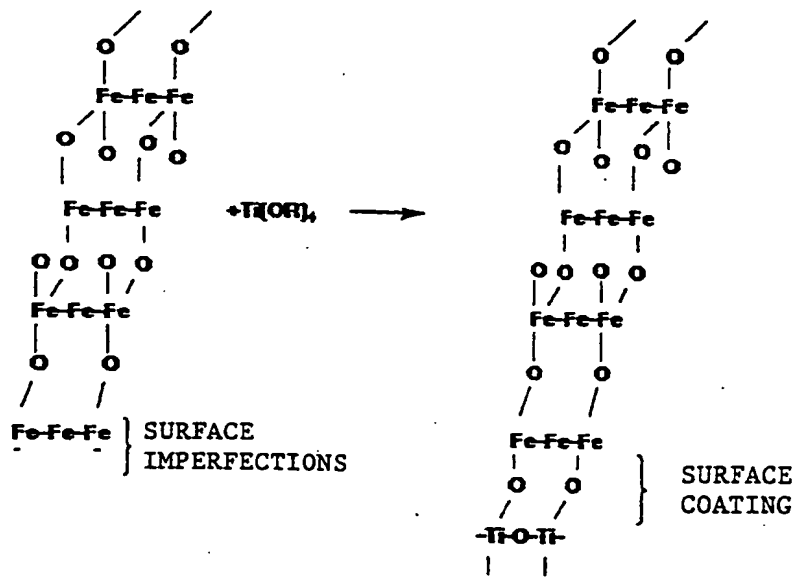
The exact size at which the onset of superparamagnetic behavior occurs in the unit crystal, is a function of the crystal structure, shape, and composition.

Several different cubic ferrites have been prepared with several different crystal sizes each. The onset of superparamagnetic behavior occurs at various size unit crystals depending on the exact composition. Table 3 is an estimate of the size where supermagnetic behavior begins for several different crystal compositions.

TABLE 3

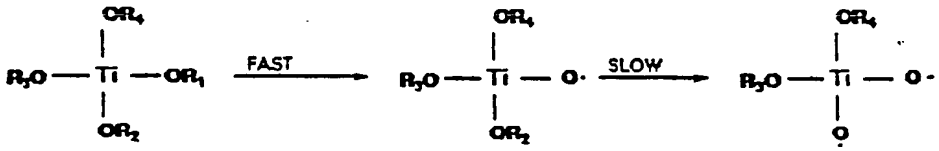
CRYSTAL COMPOSITION	MINIMUM SIZE FOR SUPERPARAMAGNETIZATION
Fe_3O_4	50A
$Fe_{2.5}Zn_{0.5}O_4$	80A
Fe_2ZnO_4	120A
$Fe_{2.5}Mn_{0.5}O_4$	100A
Fe_2MnO_4	50A
$Fe_2Sr_{0.25}Al_{0.5}O_4$	20A

The substantially uniform size Fe_3O_4 affords a crystal lattice which contains primarily trivalent iron (Fe+3) at or near the surface of the crystal. It has been found that these "surface trivalent" elements of the lattice contain imperfections which make them available for direct covalent attachment of the organo-metallic compounds of the formula $Ti(OR)_4$ according to the following general equation:



It should be noted that the imperfections of the surface trivalent iron is somewhat short-lived, and if organo-metallic coating is delayed, oxidation can occur causing the development of surface hydroxyls, which can hydrolyze, to provide an FeO coating, precluding direct covalent attachment of the organo-metallic moiety. For example, freshly made Fe_3O_4 will spontaneously react; Fe_3O_4 material after 24 hours reacts but requires about 1 hour of dwell time; after 48 hours the coupling reaction takes place very slowly and is generally incomplete.

Organo-metallic compounds are preferably of the formula $Ti(OR)_4$ wherein R is an alkyl group and the dissociation to the reactive component follows the following general reaction criterion:



Accordingly, R_1, R_2, R_3 and R_4 are selected so that rapid dissociation of the first radical (R_1) is fast, and

dissociation of subsequent radicals (R_2-R_4) is slow. It has been found that when the radicals R_1-R_4 are collectively alkyl type, the dissociation is linear with respect to the length of the chain (the shorter the chain, the faster the dissociation). Therefore, it is possible to shift the reactivity of such organo-metallic compounds by simply replacing shorter alkyl substituents with longer alkyl substitution. It has also been found that when R is an aryl moiety, dissociation is relatively slow. Other moieties (e.g. esters, ketones) have been found to provide intermediate dissociation constants.

Description of Chemical Bond Magneto Clusters

Aggregate clusters of sub 50A non-magnetic ferrites were prepared by several techniques including air drying of the particles to form agglomerates, argon drying at room temperature, several different solution encapsulation techniques and by covalent coupling of surface modified crystals. All of the techniques employed provided particle clusters of at least 250A diameter and mostly of 500A or greater. In all cases, surprisingly, the particle clusters of non-magnetic ferrite crystal were magnetic.

Organo-metallic coating with monomer material capable of adsorptive or covalent binding to iron oxide particles (of less controlled particle size) is reported in U.S. patent application no. 556,169, filed August 10, 1990. According to the instant invention, such coatings can now advantageously be applied to inorganic oxide crystal particles of substantially uniform particle size distribution. For example, substantially uniform sub 50A Fe_3O_4 was treated with titanium tetra-isopropoxide and subsequently terminated with a C-6 carboxylic acid and a second population was terminated with a C-6 amine. When mixed together and measured for magnetic response, no magnetic moment was observed. However, upon addition of methyl diisocyanate, the amine and carboxyl terminus groups spontaneously caused clustered aggregates of

magnetic particles to form and a magnetic moment proportional to the concentration of methyl diisocyanate added was observed until saturations occurred when all of the amine and/or carboxyl reagent was exhausted.

Description of the Magnetic Molecular Switch

Another application for the magnetic cluster is the so-called magneto-molecular switches. Sub 50A non-magnetic Fe_3O_4 particles are treated by mixing them in a non-aqueous solvent, such as dimethyl formamide and with titanium tri-isopropoxy-3,4-dihyroxy phenoxide.

The particles prepared in this fashion, are titanium oxide coated with o-dihyroxy benzene termination and are non-magnetic in an applied field. Upon addition of a solution of a transition metal, sodium molybdate and tungsten, for example, a 2:1 coordination complex forms between 1 metal clustered and 2 o-hydroxy benzene atoms causing the particles to become clustered and giving rise to a magnetic signal that is proportional to the concentration of metal ion coupling formed.

Surprisingly, a slight change in pH causes the complex to decompose and the resulting magnetization return to 0. A return to the pH favorable for the formation of the complex results in a renewed magnetization of equivalent field strength to that achieved after initial addition of metalate ion. This so called magneto-molecular switch is useful for, but not limited to: magnetic tracers for in vitro analysis, magnetic tracers for in vivo diagnostics, magnetic processing by metals (especially for group VI transition metals), analysis of metals, filtering aids, magneto chromatography, and cell sorting.

Description of the Applications

The inorganic oxide crystal particles of substantially uniform particle size distribution may be coupled to biological or organic molecules with affinity for or the ability to adsorb or which interact with certain other biological or organic molecules. Particles so coupled may

be used in a variety of in vitro or in vivo systems involving separation steps or the directed movement of coupled molecules to particular sites, including, but not limited to, immunological assays, other biological assays, biochemical or enzymatic reactions, affinity chromatographic purifications, cell sorting and diagnostic and therapeutic uses.

Magnetic In vitro Tracers

Controlled size inorganic oxide particles of this invention can be covalently bonded by conventional coupling chemistries to bioaffinity adsorbents including, but not limited to, antibodies (ligands, e.g., anti-thyroxine, anti-triiodothyronine, anti-thyroid stimulating hormone, anti-thyroid binding globulin, anti-thyroglobulin, anti-digoxin, anti-cortisol, anti-insulin, anti-theophylline, anti-vitamin B-12, anti-folate, anti-ferritin, anti-human chorionic gonadotropin, anti-follicle stimulating hormone, anti-progesterone, anti-testosterone, anti-estriol, anti-estradiol, anti-prolactin, anti-human placental lactogen, anti-gastrin and anti-human growth hormone antibodies), antigens (ligates, e.g. hormones, peptides, pharmacological agents, vitamins, cofactors, hematolgical substances, virus antigens, nucleic acids and nucleotides) and specific bonding proteins, which coupled particles can be used in immunassays or other binding assays for the measurement of analytes in solution. In broad aspect, when such controlled size inorganic oxide particles are non-magnetic, and bound to a given species having specific affinity for a corresponding biochemical moiety, the magnetic response becomes directly proportional to the concentration of the biochemical moiety causing the complexation.

For example, crystals are prepared that are, as explained earlier, below the critical size for the development of superparamagnetic behavior. The non-magnetic crystals are then coated with an organo-metallic

coating, for example, amino-hexyl-titanium-tri-isopropoxide, and thermally crosslinked to form an organo-titanium polymer coating having an organic spacer arm (the hexyl moiety) and organic functional group (i.e., the amino-group). Anti-T-4 (thyroid hormone) with carboxylic acid terminal functionality is then coupled to the non-magnetic crystal in the presence of CDI (carbodimide catalyst) thereby forming an amide linkage between Anti-T-4 and the coated particle. Upon the addition of T-4 hormone, clusters are formed, and magnetic properties are detected.

In a further embodiment, an antibody, such as IgG, is coupled to the non-magnetic crystals, followed by addition of antitithiophillene. Upon addition of thiophillene, magnetic clusters are formed.

In vivo Tracers

A surface modification is put on the surface of non-magnetic Fe_3O_4. The modified reagent is injected into a patient and a complex is formed at a specific site in the body. The patient is imaged by MRI, or other suitable magnetic detection techniques.

Magnetic Metal Processing/Metal Analysis

Non-magnetic Fe_3O_4 is coupled to chelating agents and put into contact with the process stream. The complex forms and gives rise to a magnetic moment on the cluster thus formed. The cluster and metal of choice are collected with a magnet. The pH is changed to strip the metal and the product is collected. For example, the non-magnetic crystals are prepared as described above, with an organo-titanium polymer coating having an organic spacer arm and a terminal amino functionality. The particles are then reacted, by and through the amino functionality, with 2,3-dihydroxy-5-benzoic acid (upon addition of CDI) to form an amide coupled product with 2,3-dihydroxy-benzene termination. When such dihydroxy functionality is brought into contact with metals such as Tu, or Mo, under

controlled pH (6-8) a complex forms and gives rise to the magnetic moment. In a similar manner, 2,3-dithio-5-benzoic acid can be employed, providing terminal dithio functionality, for more selective chelating with, e.g., Mo.

EXAMPLES

Example 1

PREPARATION OF 25A DIALYZED IRON OXIDE CRYSTAL

A stock of solution of iron salt is prepared by first dissolving 2.5g $FeCl_2.H_2O$ (Aldrich) in 37.5g of tap water at 65°C, then adding 4g $FeCl_3$ (Aldrich) to the solution and mixing until dissolved. The solution is dark orange in color. From this stock solution a dilute solution is prepared for dialysis by adding 3g of the stock iron solution to 297g of warm (50°C) water. 50g of this 1% solution is sealed in cellulose dialysis tubing (Sigma MW12000) that has been prepared in the following manner: A 12 inch strip of tubing is soaked in warm water for 30 minutes, rinsed thoroughly in warm water and stored in cool water until the addition of iron solution.

The dialysis tubing containing 50 g of the 1% iron solution is scaled and then placed in a 2% ammonium hydroxide solution:

6g NH_4OH (Ashland Chemical 28-30%) in 294g cool water

The container holding the NH_4OH solution and dialysis sack of iron solution is covered tightly and allowed to dialyze at room temperature until equilibrium is reached (4-6 hours). An orange precipitate of iron oxide forms inside the dialysis sack, white precipitate of ammonium chloride forms outside the sack. The precipitate is decanted from the tubing and washed by centrifuging, decanting the supernatant, and adding water. This step is repeated three times.

Example 2

PREPARATION OF 50A DIALYZED IRON OXIDE CRYSTAL

A stock of solution of iron salt is prepared by first dissolving 2.5g $FeCl_2.4H_2O$ (Aldrich) in 37.5g of tap water at 65°C, then adding 4g $FeCl_3$ (Aldrich) to the solution and mixing until dissolved. The solution is dark orange in color. From this stock solution a dilute solution is prepared for dialysis by adding 6g of the stock iron solution to 295g of warm (50°C) water. 50g of this 2% solution is sealed in cellulose dialysis tubing (Sigma MW12000) that has been prepared in the following manner: A 12 inch strip of tubing is soaked in warm water for 30 minutes, rinsed thoroughly in warm water and stored in cool water until the addition of iron solution.

The dialysis tubing containing 50g of the 2% iron solution is sealed and then placed in a 4% ammonium hydroxide solution:

12g NH_4OH (Ashland Chemical 28-30%) in 288g cool water

The container holding the NH_4OH solution and dialysis sack of iron solution is covered tightly and allowed to dialyze at room temperature until equilibrium is reached (4-6 hours). A dark orange precipitate of iron oxide forms inside the dialysis sack, white precipitate of ammonium chloride forms outside the sack. The precipitate is decanted from the tubing and washed by centrifuging, decanting the supernatant, and adding water. This step is repeated three times.

Example 3

PREPARATION OF 75A DIALYZED IRON OXIDE CRYSTAL

A stock solution of iron salt is prepared by first dissolving 2.5g $FeCl_2.4H_4O$ (Aldrich) in 37.5g of tap water at 65°C, then adding 4g $FeCl_3$ (Aldrich) to the solution and mixing until dissolved. The solution is dark orange in color. From this stock solution a dilute solution is prepared for dialysis by adding 9g of the stock iron

solution to 291g of warm (50°C) water. 50g of this 3% solution is sealed in cellulose dialysis tubing (Sigma MW12000) that has been prepared in the following manner: A 12 inch strip of tubing is soaked in warm water for 30 minutes, rinsed thoroughly in warm water and stored in cool water until the addition of iron solution.

The dialysis tubing containing 50g of the 3% iron solution is sealed and then placed in a 4% ammonium hydroxide solution:

12g NH_4OH (Ashland Chemical 28-30%) in 288g cool water

The container holding the NH_4OH solution and dialysis sack of iron solution is covered tightly and allowed to dialyze at room temperature until equilibrium is reached (4-6 hours). A brown precipitate of iron oxide forms inside the dialysis sack, while precipitate of ammonium chloride forms outside the sack. The precipitate is decanted from the tubing and washed by centrifuging, decanting the supernatant, and adding water. This step is repeated three times.

Example 4

SYNTHESIS OF TITANIUM COATED 100A MAGNETIC PARTICLES

Titanium coated magnetite, Fe_3O_4, is prepared using the following method:

Iron salts, $FeCl_2.4H_2O$ and $FeCl_3$ (41g) are each dissolved in 1000 cc of water. The solutions are combined into a 2 liter beaker and 70 ml of ammonium hydroxide is added while mixing. The beaker containing the resulting precipitate, 28 gm of Fe_3O_4, is then placed onto a permanent magnet to magnetically separate the magnetic particle from the salt by-products. After resting on the magnet for 5 minutes, the clear salt solution is decanted. The precipitate is then resuspended in a total of 1500 cc of water and placed on a permanent magnet for 5 minutes before decanting. The above washing process is repeated three additional times. After the final decanting, the

magnetite is suspended in 1500 cc of dry acetone and magnetically separated as above. The particles are acetone washed a total of 3 times. After the final decanting, the particles are suspended in 500 cc of N,N dimethyl formamide.

The solution, 250 cc, is poured into a horizontal bead motor mill and milled for 10 minutes to ensure efficient dispersion. Titanium isopropoxide, 35 gm, dissolved in 50 cc of N,N dimethyl formamide is slowly pipetted into the funnel of the operating motormill and milled for 15 minutes.

The dispersion is removed from the mill, magnetically separated, decanted and water washed 5 times with 1000 cc of distilled water.

Example 5

SYNTHESIS OF TITANIUM COATED 20A NON MAGNETIC PARTICLES

This example illustrates the preparation of organometallic, titanium isopropoxide, coated non-magnetic 20A ferrites. A dispersion of non-magnetic 20A particles is water washed five times and anhydrous methanol washed three times by centrifugation. A total of 5.0 g of particle is suspended in 250 ml of N,N dimethyl formamide and milled in a bead motormill for 15 minutes. 12.0g titanium isopropoxide dispersed in 30.0 g N,N- dimethyl formamide is slowly pipetted into the operating mill and milled for another 15 minutes. The product is then removed to form the mill and water washed five times by centrifugation and resuspended in distilled water.

Example 6

SYNTHESIS OF AMINE TERMINATED MAGNETIC PARTICLES

Magnetite coated with an organometallic, Ti, and terminated with a C-6 amine is prepared using the following method.

The precipitation, washing and coating with organometallic, titanium isopropoxide, is conducted in the exact manner as described above. After the washed

magnetite particle, N,N- dimethyl formamide and titanium isoproxide have milled for 15 minutes, 15 gm of 6-amino 1-hexanol dissolved in 30 cc of N,N dimethyl formamide is pipetted into the operating mill. After milling for 15 minutes, the dispersion is heated for 20 minutes at 100°C with occasional mixing. The dispersion is then allowed to cool, magnetically separated and washed five times with 1,000cc of distilled water.

Example 7

SYNTHESIS OF CARBOXYL TERMINATED MAGNETIC PARTICLES

Magnetite coated with an organometallic, Ti, and terminated with a C_6 carboxyl group is prepared as follows:

14.2 g of 4-hydroxy butyric acid sodium salt dispersed in 30 cc of N,N-dimethyl formamide is slowly pipetted to the 250 cc of washed organometallic coated magnetic particles as described above in Example 4. After milling for 15 minutes, the dispersion is heated for 20 minutes at 100°C with mixing. The solution, at room temperature, is magnetically separated and washed five times with 1,000 cc of distilled water.

Example 8

SYNTHESIS OF DIHYDROXY AROMATIC TERMINATED MAGNETIC PARTICLES

This example illustrates the preparation of dihydroxy-aromatic terminated magnetic particle. 5 g of magnetite coated with titanium isopropoxide and 6-amino-1-hexanol, prepared as above, is dispersed in sodium metabisulfite and distilled water solution, 300 cc. The sodium metabisulfite solution has been pretreated with nitrogen gas to prevent oxidation of the particles. 78 g of gallic acid, and 1.0 g of carboddimide is combined with the amine-terminated magnetic particle with mixing. After incubating for one hour, the product is magnetically separated and water washed.

Example 9

MAGNETIC TRACERS FOR IMMUNO ASSAY I

20A non-magnetic ferrite particles were washed 4 times with water, 3 times with acetone and 3 times with anhydrous methanol by collecting the particles after centrifugation and resuspending the particles by vigorous agitation.

Tyzor (titanium tetra-isopropoxide), dissolved in anhydrous methanol was added to 0.53 g dry of particles at 25 g Ti/9.6 g dry particles. Steel balls were added and the particles were milled in a ball mill for one hour.

The particles were then amine terminated by adding 6-amino-1-hexanol dissolved in anhydrous methanol to the Tyzor coated particles. For every 9.6 g dry particles, .088 mol amine was used. This was added to the Tyzor coated particles and milled on the ball mill for 3 1/2 hours. The magnetics were tested on a vibrating sample magnetometer. The particles were found to be non-magnetic.

The sample was divided into 4 equal dry parts of 0.13 g each. 1,6 diisocyanato-hexane was added to particles in four concentrations: 0, .5, 4, 8 lm 1,6 diiso./.5 g dry. The particles were milled overnight in the ball mill without using steel balls.

The magnetics were tested again on the VSM. It was determined that the increase in 1,6 diisocyananatohexane resulted in a proportional increase in magnetivity.

Example 10

ENCAPSULATION BY A POLYMER

20A non-magnetic ferrite particles were washed 4 times with water, 5 times with acetone, (collecting with a centrifuge between washes). The acetone slurry is then washed 5 times with hexane. A solvent borne solution of the polymer (e.g., polystyrene, polyurethane, poly(vinyl chloride)) from about 0.1%-10% by weight in an amount equal to about 1:10 to 10:1 particle:polymer ratio is then

added. Mixing continues for about 10 minutes in a high shear mixer to allow the crystals to coat uniformly with polymer. Water is then added in a volume equal to about 10-100 times the amount of solvent to flocculate the polymer. The beads are then collected. In the case of polyurethane, it has been found the THF is the solvent of choice.

Example 11

ADDITION OF MONOMER FOLLOWED BY CROSSLINKING

A particle slurry is prepared as in Example 10. Oleic acid is then added to the hexane slurry of particles and mixed in a high shear mixer for about 20 minutes. A volume of acetone is then added, equal to approximately 5 times the amount of hexane to the oleic acid coated particle dispersion, in order to flocculate. The resulting residue is collected and mixed in water in a high shear mixer for about 1 hour to produce oleic acid coated crystal beads. The bead slurry is then exposed to 3-beam generator (Energy sources, Woburn, MA), from 1-20 meg Rad for about 0.25-0.5 sec., to crosslink through the unsaturated group.

Example 12

PREPARATION OF SUB 10 NM PARTICLES IN A TWO-SIDED DIALYSIS TANK

2 nm diameter uniform magnetic crystals were prepared by controlled contact of a base solution and iron salt solution across a semipermeable membrane, resulting in an iron oxide crystal precipitate of defined size within a narrow size distribution range. A Spectra/Por® 5 dialysis membrane (flat sheet) was affixed in a manner as to separate two equal sized chambers of a two sided Dialysis reaction tank. Both sides of the tank were filled with 20 liters of distilled H_2O at 20°C. 12.5g $FeCl_2$ $4H_2O$ and 20g $FeCl_3$ were added to one chamber of the tank and stirred until dissolved. 60.6g NaOH were dissolved in 2 liters of H_2O and added to the solution into the opposite chamber in

the tank. Both sides were agitated by a mechanical paddle stirrer for 15 min. After 70-80 hours of contact time, the iron solution and precipitated crystals were removed from the tank and the magnetic crystals were collected by centrifugation and measures by TEM to be 2 nm average diameter.

Uniform size inorganic core particles can be prepared by the preferred method reported in U.S. Patent Application Serial No. 894,260, filed June 8, 1992, the teachings of which are incorporated by reference. As described therein, aqueous solutions of an inorganic salt and an inorganic base are contacted across a porous membrane wherein the membrane contains a plurality of pores which allows for precipitation of substantially monodispersed inorganic oxide particles on one side of the membrane and precipitation of a salt of the corresponding base on a second side of the membrane. Particle size diameter can range between 5-1000 Angstroms, and in a preferred embodiment, 5-100 Angstroms, with a particle size distribution of +/- 10%. The inorganic salts are of the formula MY, wherein M is selected from the group consisting of Fe, Co, Ni, Zn, Mn, Mg, Ca, Ba, Sr, Cd, Hg, Al, B, Sc, Ga, V, In, and mixtures thereof, with Y being selected from the group consisting of Cl, Br, I, SO_4, NO_3, PO_4 and mixtures thereof. The inorganic base is selected from the group consisting of NH_4OH, KOH, LiOH, NaOH, CsOH, RbOH and mixtures thereof. Accordingly, and in a preferred embodiment, Fe_3O_4 is prepared (a mixed Fe(+2)Fe(+3) oxide of the formula $[Fe(+2)][Fe(+3)]_2O_4$) with a uniform sub 100 Angstroms diameter serving as the inorganic core of the liposomes described herein.

Inorganic core particles can also be prepared according to the following general procedure: metal salts, or organometallocenes are precipitated in base at high temperature and pressure to form fine magnetic metal oxide crystals. The crystals are redispersed, then washed

in water and an electrolyte. Magnetic separation can be used to collect the crystal between washes. The crystals are then milled to a more controlled particle size, for example, in a ball mill, under conditions sufficient to form 50 Angstroms or lower particle size. See, U.S. Patent No. 5,071,076, and U.S. Patent Application Serial No. 806,478, filed December 31, 1991, the teachings of which are incorporated by reference.

III. Amphipathic Organic Compounds

The amphipathic organic compounds which can be used in forming the inorganic core liposome of the invention may be selected from a variety of organic compounds which contain both a hydrophobic and hydrophilic moiety. According to one important aspect of the invention, it has been discovered that the hydrophilic moiety is adsorbed or coordinated onto the surface of the inorganic oxide, whereas the hydrophobic moiety of the molecule extends outwardly to associate with the amphipathic vesicle forming lipid compounds. Preferred amphipathic organic compounds include fatty acids selected from the group consisting of oleic, stearic, linoleic, lionlenic, palmitic, nyristic and arachidonic acid.

IV. Amphipathic Vesicle Forming Lipid Components

The lipid components used in forming the inorganic core liposomes of the invention may be selected from a variety of vesicle forming lipids, typically including phospholipids, such as phosphatidylcholine (PC), phosphatidic (PA), phosphatidylinositol (Pl), sphinogomyelin (SM), and the glycolipids, such as cerebroside and gangliosides. The selection of lipids is guided by consideration of (a) drug release rate is serum, (b) drug-entrapment efficiency, (c) liposome toxicity, and (d) biodistribution and targeting properties. A variety of lipids having selected chain compositions are commercially available or may be obtained by standard

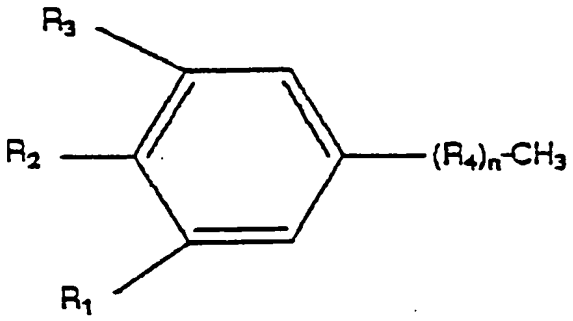
lipid isolation procedures. See, e.g. U.S. Patent No. 4,994,213.

The lipids may be either fluidic lipids, e.g. phospholipids whose acyl chains are relatively unsaturated, or more rigidifying membrane lipids, such as highly saturated phospholipids. Accordingly, the vesicle forming lipids may also be selected to achieve a selected degree of fluidity or rigidity to control the stability of the liposome in serum and the rate of release of entrapped drug from the liposome in the bloodstream. See, e.g. U.S. Pat. No. 5,013,556.

In a preferred embodiment, the vesicle forming lipid include those phospholipids in which the polar-head-group region is modified by the covalent attachment of polyalkylene ether polymers of various molecular weights. The attachment of the relatively hydrophilic polyalkylene ether polymer, particularly polyethylene oxide, alters the hydrophilic to hydrophobic balance within the phospholipid in order to give unique solubility to the phospholipid compound in an aqueous environment. See, e.g. U.S. Pat. No. 4,426,330. The polyalkyl ether lipid is preferably employed in the inorganic core liposome composition in an amount between about 1-20 mole percent, on the basis of moles of derivatized lipid as a percentage of total moles of vesicle-forming lipids. The polyalkylether moiety of the lipid preferably has a molecular weight between about 120-20,000 daltons, and more preferably between about 1000-5000 daltons.

In yet another embodiment of the present invention, a new series of phenyl lipid compounds are described which

have the following structural formula:



wherein two of R_1, R_2 and R_3 represent a saturated or unsaturated straight-chain or branched chain hydrocarbon group, the other being hydrogen, therein providing at least two hydrocarbon chains attached to the phenyl moiety, wherein the two hydrocarbon chains are typically between about 14-22 carbon atoms in length, and have varying degrees of unsaturation. R_4 represents the repeating unit of either a poly(alkylene oxide) polymer, preferably ethylene, propylene and mixtures thereof, or the repeating unit of poly(vinyl alcohol). The number of alkylene oxide or vinyl alcohol groups in the polymer, designated as n, may vary from 0 to about 200 or more.

V. Preparing the Inorganic Core Liposome Composition

One preferred method for producing the uniform size inorganic core liposome composition begins with first coating the magnetic particles described above in Section

II with an amphipathic organic compou[nd] which contains both a hydrophillic and hydrophobic m[oie]ty. For example, fatty acids, such as oleic acid, lino[leic] acid or linolenic acid, dispersed in an organic solvent, are directly added to the particles at a ratio of dry Fe_3O_4:acid equal to 2:1 weight percent. After mechanically milling the mixture for 1 to 1.5 hours on a ball mill with 4 mm glass media, the acid coated particles collapse around the media allowing for easy removal of water without the loss of the particles. The coated particles are then dispersed in an organic solvent by addition of 700 ml of hexane, toluene or chloroform and mechanically milling with glass media overnight (15 hrs).

Absorbing a phospholipid onto the fatty acid coated particles was accomplished by addition of a synthetic polyethylene glycol terminated phosphatidyl ethanolamine to the above dispersion and mechanically mixing for 3 hours. The ratio of fatty acid:pure lipid is about 1:1 weight percent.

To transfer the particles from an organic phase to an aqueous phase, 7 mls of the dispersion was placed into a 14 ml glass vial with 3 ml of distilled water. The vial was placed in warm, 35°C sonicating water bath with N_2 bubbling through it to evaporate the solvent. Once the solvent has evaporated, the aqueous dispersion was then suspended in a total of 10 mls of autoclaved water, sonicated for one hour, and centrifuged for 5 minutes. The supernatant was removed and brought to 20 mg particle/ml solution with autoclaved water.

VI. Utility

From the above, it can be appreciated that the present invention offers a number of advantages over prior art liposome-methods. The preparation of uniform size inorganic core particles by dialysis and precipitation across a semi-permeable membrane is unique in its ability to allow for the production of uniform size liposomes

without the requirement for extrusion or other additional liposome sizing techniques. The ability to selectively vary the average size of liposomes, according to lipid composition and/or ionic strength, is another useful feature of the invention. While the present invention provides inorganic core liposomes with a size range of about 5-5000 nm, one selected size range, between about 100-300 nm, is particularly useful for a variety of parenteral uses, as discussed.

One general class of drugs include water-soluble liposome permeable compounds which are characterized by a tendency to partition preferentially into the aqueous compartments of the liposome suspension, and to equilibrate, over time, between the inner liposomal spaces and outer bulk phase of the suspension. Representative drugs in this class include terbutaline, albuterol, stropine methyl nitrate, cromolyn sodium, propracalol, funoisolide, ibuprofin, geniamycin, tobermycin, pentamidine, penicillin, theophylline, bleomycin, etopoxide, captoprel, n-acetyl cystein, verapamil, vitamins, and radio-opaque and particle-emitter agents, such as chelated metals. Because of the tendency of these agents to equilibrate with the aqueous composition of the medium, it is preferred to store the liposome composition in lyophilized form, with rehydration shortly before administration.

A second general class of drugs are those which are water-soluble, but liposome-impermeable. For the most part, these are peptide or protein molecules, such as peptide hormones, enzymes, enzyme inhibitors, apolipoproteins, and higher molecular weight carbohydrates characterized by long-term stability of encapsulation. Representative compounds in this class include calcitonin, atriopeptin, -1 antitrypsin (protease inhibitor), interferon, oxytocin, vasopressin, insulin, interleukin-2, superoxide dismutase, tissue plasminogen activator (TPA),

plasma factor 8, epidermal growth factor, tumor necrosis factor, lung surfactant protein, interferon, lipocortin, α -interferon, macrophage colony stimulating factor, and erythroprotein.

A third class of drugs are lipophilic molecules. The drugs in this class are defined by an oil/water partition coefficient, as measured in a standard oil/water mixture such as octanol/water, of greater than 1 and preferably greater than about 5. Representative drugs include prostaglandins, amphotericin B, progesterone, isosorbide dinitrate, testosterone, nitroglycerin, estradiol, doxorubicin, epirubicin, beclomethasone and esters, vitamin E, cortisone, dexamethasone and esters, and betamethasone valerete.

In another application, the inorganic core liposome composition is designed for targeting a specific target tissue or organ. For example, this feature allows for targeting a tumor tissue, for drug treatment by intravenous administration to a tumor-bearing subject.

As another example, the inorganic core liposomes may be prepared with surface-bound ligand molecules, such as antibodies, which are effective to bind specifically and with high affinity to ligand-binding molecules such as antigens, which are localized specifically on target cells.

A variety of methods for coupling ligands to the surface of liposomes are known, including the incorporation of ligand-derivatized lipid components into liposomes or coupling of ligands to activated liposome surface components.

The targeted inorganic core liposomes may be prepared to include cancer chemotherapeutic agents, such as those listed above. In one preferred embodiment, the liposomes are prepared to include PEG-PE and PG, to a final

concentration of charged lipids up to 40 mole percent, doxorubicin, and remainder neutral phospholipids or neutral phospholipids and cholesterol.

In an inorganic core liposome composition which is useful for radio-imaging of solid tumor regions, the liposomes are prepared with encapsulated radio-opaque or particle-emission metal, typically in a chelated form which substantially prevents a permeation through the liposome bilayer.

In still another application, the liposome composition is designed to enhance uptake of circulating cells or other blood-borne particles, such as bacteria, virus-infected blood cells and the like. Here the long-life liposomes are prepared to include surface-bound ligand molecules, as above, which bind specifically and with high affinity to the selected blood-borne cells. Once bound to the blood-borne particles, the liposomes can enhance uptake by the RES.

Polyalkylether moieties on the liposomes may be derivatized by the associated amphipathic lipid by an ester, peptide, or disulfide bond which can be cleaved, after liposome binding, to the target cells, to further enhance RES particle clearance.

Studies performed in support of the present invention indicate that the inorganic core liposome composition of the invention provides an enhancement in blood circulation lifetime which is equal, and in some cases superior, to the most effective RES-evading rigid-lipid liposomes which have been reported heretofore, including liposomes containing GMI and membrane-rigidifying lipids.

The blood circulation lifetimes achieved in the present invention should be substantially greater than with fluid-core liposomes.

The following examples illustrate methods of preparation of inorganic core liposomes with enhanced circulation times, and for accessing circulation times in

vivo and invitro. The examples are intended to illustrate specific inorganic-core liposome compositions and methods of the invention, but are in no way intended to limit the scope thereof.

DESCRIPTION OF THE EMBODIMENTS

EXAMPLE 1

Preparation of Magnetic Particles by Co-precipitation of Fe+2/Fe+3 with Excess Base

Magnetic particles of 100 Angstroms in diameter are prepared using the following method. Iron salts, $FeCl_2^-$, $3H_2O$, (25g), and $FeCl_3$ (41g) are each dissolved in 1000 cc of water. The solutions are combined into a 2 liter beaker and 70ml of ammonium hydroxide is added while mixing. The resulting black magnetic precipitate yields 28gm of magnetite, Fe_3O_4.

EXAMPLE 2

Preparation of sub 10 nm particles

2 nm diameter uniform magnetic crystals were prepared by controlled contact of a base solution and iron salt solution across a semipermeable membrane, resulting in an iron oxide crystal precipitate of defined size within a narrow size distribution range.

A Spectra/Por 5 dialysis membrane (flat sheet) was affixed in a manner as to separate two equal sized chambers of a two sided Dialysis reaction tank. Both sides of the tank were filled with 20 liters of distilled H_2O at 20°C. 12.5 g $FeCl_2^-$ $4H_2O$ and 20g $FeCl_3$ were added to one chamber of the tank and stirred until dissolved. 60.6g NaOH were dissolved in 2 liters of H_2O and added to the solution into the opposite chamber in the tank. Both sides were agitated by a mechanical paddle stirrer for 15 min. After 70-80 hours of contact time, the iron solution and precipitated crystals were removed from the tank and the magnetic crystals were collected by centrifugation and measures by TEM to be 2nm average diameter.

EXAMPLE 3

Preparation of Oleic Acid Coated Magnetite

Magnetic particles, Fe_3O_4, coated with oleic acid are prepared using magnetite as precipitated in Example 1. The magnetite is water washed by successive additions of distilled water to a slurry concentrate of magnetite. The beaker containing the magnetite slurry is place onto a permanent magnet to magnetically separate the magnetic particle from the salt by-products between each successive addition of water. After resting the slurry on the magnet for 5 minutes, the aqueous salt solution is decanted. The precipitate is then resuspended with agitation in a total of 1500 cc of water and placed on a permanent magnet for 5 minutes before decanting. The above washing process is repeated three additional times with water. After the final water wash is decanted, the particles are acetone washed and hexane washed a total of 5 times each in the above manner.

Oleic acid is added to the magnetic hexane slurry in a ratio of oleic acid:dry particle equal to 2:1 weight percent. The mixture is adjusted to 15% total solids with hexane and mechanically milled overnight in a glass jar half filled with 3mm stainless steel media.

EXAMPLE 4

Preparation of Oleic Acid Coated Dialyzed Magnetic Particles

Dialyzed particles coated with oleic acid are prepared using particles as prepared in Example 2. 0.1 grams of particles are washed with three 200 ml volumes of distilled water and acetone by suspending approximately 0.1gm dry particle in 200 ml of acetone and centrifuging for 45 minutes to collect particles between each washing.

Oleic acid was added to the acetone slurry in a ratio of oleic acid:dry particle equal to 2:1 weight percent and mechanically milled overnight in a glass jar half filled with 3mm glass media.

EXAMPLE 5

Preparation of Magnetite Core Liposomes using Phosphatidyl Choline

10 gms Oleic acid coated magnetite as prepared in Example 3 was dispersed in 100 cc hexane. The phosphate lipid is absorbed onto the particle by dissolving phosphatidyl choline (Sigma, P-3644, L-2, lechithin, 45% PC) into hexane with heating to create a 15% solution. The PC/hexane solution is combined with the magnetic/hexane solution at a ratio of pure phosphatidyl choline:oleic acid equal to 1:2 weight percent.

The solution was mixed in a glass jar (without media) on a jar roller for two hours. After mixing, the lipid was absorbed onto the particle by adding three times as much acetone than hexane and collecting the lipid coated particles over a magnet. After the coated magnetic particles were separated from the solvents, the solvents were decanted, distilled water was added to produce a 2.0% TS slurry. The slurry is heated in a beaker on a hot plate to 100°C for 10 min. From 0.5 to 50 grams of triton x-114 (Union Carbide) was added to disperse the lipidized magnetic particles in an aqueous system. A ratio of triton x114:lipid particle equal to 1:6 weight percent was the optimum level for the dispersion. The dispersion was mixed on a laboratory vortex mixer for 2 minutes and placed in an ultrasonic bath (Branson 1200, VWR) for hours. The final dispersion is adjusted to 0.2% TS (2mg/ml). Particles were measured on a Nycomp laser particle size analyzer and were found to be approximately 200 nm in diameter.

EXAMPLE 6

Preparation of Phenyl Lipid

A. Synthesis of a m-isophthalic acid based phenyl lipid.

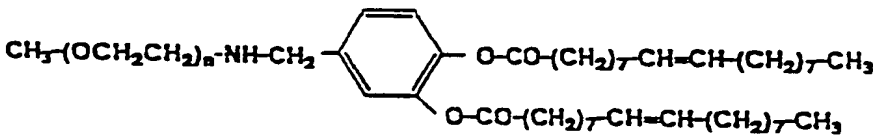
The starting material for this synthesis if 5-Aminoisophthalic acid. The 5-aminoisophthalic acid is not soluble in dioxane alone. It is soluble in a mixture

of dioxane and triethylene glycol. 5-aminoisophthalic acid (145 mg) was dissolved in 5 ml. of dioxane and 2 ml. of triethylene glycol, and the pH was adjusted to 10 with NaOH. Methoxypolyoxyethylene imidazoly carbonyl, average mol. wt. 5,000 from Sigma (2.0g) was dissolved in 2ml of H_2O, 1.0ml of 1N Na_2CO_3, and 2.0 ml of triethylene glycol. This solution was added to the 5-aminoisophtlalic acid solution and stirred for 36 hours at room temperature. The reaction mixture was then dialyzed overnight against 2 liters of H_2O. The dialyzed reaction mixture was mixed with 100ml of pyridine and the liquids removed via rotary evaporation. The resulting yellow oil was placed in the refrigerator. After several days a white precipitate formed. The precipitate contains both coupled and uncoupled PEG.

Oleyl alcohol can be coupled to the above isophthalic acid derivative using thionyl chloride. The thionyl chloride can be used to activate the oleyl alcohol for ester formation with the carboxyl groups of the isophthalate. See. Fig. 2.

B. Synthesis of ortho phenyl lipids

The ortho analog of the phenyl lips can be synthesized starting with either 3,4 dihydroybenzaldehyde or 3,4 dihydroxybenzoic acid. The aldehyde group can be coupled to an amino group by forming the Schiff's base and then reducing it with $NaBH_4$. Olegic acid could then be coupled to the hydroxyl groups using thionyl chloride to provide:



3,4 dihydroxybenzolic acid could be coupled through its carboxyl group to amino-terminated PEG using

dicyclohexyl carbodiimide. Oleic acid could then be coupled as above.

Since both amino and carboxyl PEG derivatives as well as both oleic acid and oleylamine are available, the PEG and oleic acid groups can be easily interchanged in the above compounds.

VII. Preparation of Wave Absorbing Magnetic Core Particles

The wave absorbing magnetic core particles suitable in the present invention are those particles which, upon application of an electromagnetic field, create inductive heat local to the particle. In a preferred embodiment, the wave absorbing magnetic core particles comprise ferrite or mixed ferrite materials, preferably of a uniform, controllable size, and narrow size distribution, wherein the primary component, the oxide, is of the formula $M_2(+3)M(+2)O_4$, wherein M(+3) is Al, Cr or Fe, and M(+2) is Fe, Ni, Co, Zn, Ze, Ca, Ba, Mg, Ga, Gd, Mn or Cd. In a further aspect, the oxides can be advantageously mixed with LiO, NaO and KO, or with α or γ Fe_2O_3 and Fe_3O_4.

The preparation of substantially uniform size oxides, 1 to 50,000 nm in diameter, is achieved by conversion of hydrous oxide gels, in a multi-step process, wherein alkali is added to individual M(+3) and M(+2) aqueous solutions, which separately precipitate the corresponding metal hydroxide. The two precipitates are then coarsely mixed to provide micron size amorphorus gel particles, which can be milled to form hydrous oxide gel particles about 100 A in diameter. These particles are then heated to effect dehydration, in the presence of oxygen or air, wherein the dehydration temperature, time of dehydration, and concentration of oxygen or air operate to control the particle size of the oxide crystals therein produced.

For example, in connection with the above, a dehydration temperature of 100°C, at a time of about 6 hours, in the presence of oxygen, provides oxides particles of about 70A diameter. Alternatively, a dehydration temperature of about 65°C, at a time of about 24-36 hours, in the presence of oxygen, affords oxide particle sizes of about 1000-2000A. Accordingly, by recognizing that short dwell times and high temperature promote small oxide particle formation, and that long dwell times and low temperature promote large particle formation, oxide particles from 50A to several microns in diameter have been produced.

Heretofore, the use of ferrite materials as a protective medium for electromagnetic radiation reflecting surfaces was well known. In the present invention, however, it has been found that very small ferrospinal particles provide a high degree of absorbtion of electromagnetic waves. It has also been found that the complex permeability of certain ferromagnetic metallic oxides varies with frequency in such a way as to provide high absorption of electromagnetic magnetic radiation over wide frequency ranges without using large amounts of absorber material. Upon exposure to electromagnetic waves, these ferrites generate significant infra-red radiation over short distances local to the ferrite particle's surface.

In general, those ferrites suitable for use in the present invention are cubic crystalline materials characterized by a spinal structure containing Fe_2O_3 and at least one other oxide, usually of a bivalent metal, e.g. lithium oxide, cadmium oxide, nickel oxide, iron oxide and zinc oxide.

The ferrite materials of this invention can also be prepared by a thermal process, in which they are mixed together then ground together mixed and fired at about 1200°C in a tube furnace for four hours or made by

oxidation of ferrite powders from metal hydroxide gels. The imaginery permeability must be high enough to produce a large loss. For high frequencies, it has been found that nickel can replace lithium and for narrow bands zinc can replace cadnium.

One preferred mixed ferrite having the composition 0.45 LiO, 0.5 Fe_2O_3 + 0.30 $CdFe_2O_4$ + 0.25 Fe_3O_4 yielded the following results:

Frequency Range (mHz)	% Absorbance	Surface Temp
1800-2500	98	230

As noted above, ferrites of interest to this invention can also be prepared by conversion of hydrous oxide gels in a multi-step process. In one particular preferred example, alkali is added to a ferrous sulphate solution at a temperature between 15 and 40°C, in a stoichiometric amount adapted to precipitate ferrous hydroxide, from the Fe++ ion. At the conclusion of said precipitation, air is blown into the slurry, thus oxidizing ferrous hydroxide to goethite, FeO(OH).

During a second step, alkali is added to the slurry obtained in the first step. The remaining Fe++ is precipitated in the form of ferrous hydroxide, and the slurry is heated to a temperature between 70°C and 100°C thus causing the formation of ferrite which is then separated from the solution.

Accordingly, the present invention provides a process suitable for treating ferrous sulphate solutions in order to obtain ferrite exhibiting an equiaxial morphology with a narrow particle size distribution.

VIII. Amphipathic Organic Compounds

The amphipathic organic compounds which can be used in forming a liposome composition comprising the wave

absorbing magnetic core particle may be selected from a variety of organic compounds which contain both a hydrophobic and hydrophilic moiety. According to one important aspect of the invention, it has been discovered that the hydrophilic moiety is adsorbed or coordinated onto the surface of the wave adsorbing magnetic core particle, whereas the hydrophobic moiety of the molecule extends outwardly to associate with amphipathic vesicle forming lipid compounds.

When the wave absorbing magnetic core particle is freshly made Fe_3O_4, it has been found, as reported in U.S. Patent Application Serial No. 894,260, filed June 8, 1992, that surface trivalent elements of the core particle contain imperfections which makes them available for direct covalent attachment with organometallic compounds of the formula $Ti(OR)_4$, wherein R is an alkyl group. Accordingly, the wave absorbing magnetic core particle can be coated with an organometallic coating covalently bonded to said particle wherein the bonding does not depend upon hydroxy functionality on the surface of said particle. Such coated particles can then be associated with an amphipathic vesicle forming lipid.

Preferred amphipathic organic compounds include fatty acids selected from the group consisting of oleic, stearic, linoleic, linolenic, palmitic, myristic and arachidonic acid.

IX. Amphipathic Vesicle Forming Lipid

The lipid components used in forming the wave absorbing magnetic core particle liposomes of the invention may be selected from a variety of vesicle forming lipids, typically including phospholipids, such as phosphatidylcholine (PC), phosphatidic (PA), phosphatidylinositol (PI), sphinogomyelin (SM), and the glycolipids, such as cerebroside and gangliosides. The selection of lipids is guided by consideration of liposome toxicity and biodistribution and targeting properties. A

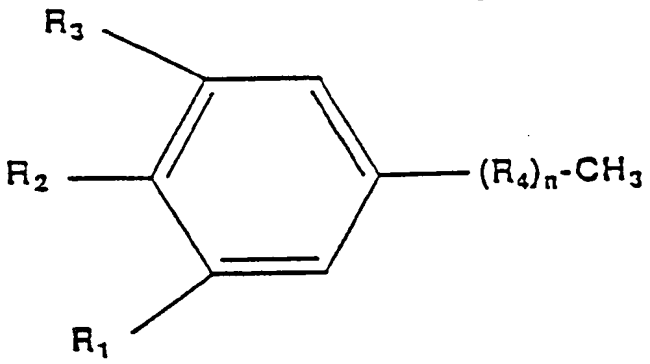
variety of lipids having selected chain compositions are commercially available or may be obtained by standard lipid isolation procedures. See, e.g. U.S. Patent No. 4,994,213.

The lipids may be either fluidic lipids, e.g. phospholipids whose acyl chains are relatively unsaturated, or more rigidifying membrane lipids, such as highly saturated phospholipids. Accordingly, the vesicle forming lipids may also be selected to achieve a selected degree of fluidity or rigidity to control the stability of the liposome in serum. See, e.g. U.S. Pat. No. 5,013,556.

In a preferred embodiment, the vesicle forming lipid include those phospholipids in which the polar-head-group region is modified by the covalent attachment of polyalkylene ether polymers of various molecular weights. The attachment of the relatively hydrophilic polyalkylene ether polymer, particularly polyethylene oxide, alters the hydrophilic to hydrophobic balance within the phospholipid in order to give unique solubility to the phospholipid compound in an aqueous environment. See, e.g. U.S. Pat. No. 4,426,330. The polyalkyl ether lipid is preferably employed in the wave absorbing magnetic core particle liposome composition in an amount between about 1-20 mole percent, on the basis of moles of derivatized lipid as a percentage of total moles of vesicle-forming lipids. The polyalkylether moiety of the lipid preferably has a molecular weight between about 120-20,000 daltons, and more preferably between about 1000-5000 daltons.

In yet another embodiment of the present invention, phenyl lipid compounds (as reported in U.S. Application Serial No. 958,646) can be employed as amphipathic vesicle forming lipid components. These phenyl lipids have the

structural formula:



wherein two of R_1, R_2 and R_3 represent a saturated or unsaturated straight-chain or branched chain hydrocarbon group, the other being hydrogen, therein providing at least two hydrocarbon chains attached to the phenyl moiety, wherein the two hydrocarbon chains are typically between about 14-22 carbon atoms in length, and have varying degrees of unsaturation. R_4 represents the repeating unit of either a poly(alkylene oxide) polymer, preferably ethylene, propylene and mixtures thereof, or the repeating unit of poly(vinyl alcohol), or a polycarbohydrate. The number of alkylene oxide or vinyl alcohol groups in the polymer, designated as n, may vary from 0 to about 200 or more.

X. Preparing the Wave Absorbing Magnetic Core Particle Liposome Composition

One preferred method for producing the wave absorbing magnetic core liposome composition begins with first coating the magnetic particles described above in Section II with an amphipathic organic compound which contains both a hydrophillic and hydrophobic moiety. For example, fatty acids, such as oleic acid, linoleic acid or linolenic acid, dispersed in an organic solvent, are directly added to the particles at a ratio of dry ferrite:acid equal to 2:1 weight percent. After mechanically milling the mixture for 1 to 1.5 hours on a

ball mill with 4 mm glass media, the acid coated particles collapse around the media allowing for easy removal of water without the loss of the particles. The coated particles are then dispersed in an organic solvent by addition of 700 ml of hexane, toluene or chloroform and mechanically milling with glass media overnight (15 hrs).

Absorbing a phospholipid onto the fatty acid coated particles was accomplished by addition of a synthetic polyethylene glycol terminated phosphatidyl ethanolamine to the above dispersion and mechanically mixing for 3 hours. The ratio of fatty acid:pure lipid is about 1:1 weight percent.

To transfer the particles from an organic phase to an aqueous phase, 7 mls of the dispersion was placed into a 14 ml glass vial with 3 ml of distilled water. The vial was placed in warm, 35°C sonicating water bath with N_2 bubbling through it to evaporate the solvent. Once the solvent has evaporated, the aqueous dispersion was then suspended in a total of 10 mls of autoclaved water, sonicated for one hour, and centrifuged for 5 minutes. The supernatant was removed and brought to 20 mg particle/ml solution with autoclaved water.

XI. Utility

The targeted wave absorbing magnetic core liposome may be prepared to include ferrites useful as cancer chemotherapeutic agents. In one method of synthesis, the magnetic core liposomes are prepared to include PEG-PE and PG on the liposome backbone to aid in targeting to specific areas and to avoid RES uptake.

Magnetic liposome compositions are also useful for radio-imaging or MRI imaging of solid tumor regions prior to EM wave exposure and cell destruction. The magnetic liposomes are prepared with encapsulated radio-opaque or particle-emission metal oxides or ferrites which

substantially prevents permeation through the magnetic liposome bilayer.

In still another application, the magnetic liposome composition is designed to enhance uptake of circulating cells or other blood-borne particles, such as bacteria, virus-infected blood cells and the like. Here the long-life magnetic liposomes are prepared to include surface-bound ligand molecules, as above, which bind specifically and with high affinity to the selected blood-borne cells. Once bound to the blood-borne particles, the magnetic liposomes can be exposed to EM fields for specific cell or virus destruction.

Other objects and advantages of this invention will become apparent upon consideration of the following working examples.

EXAMPLE 1

Preparation of Absorbing Ferrite by Thermal Processes

A mixture consisting of nickel oxide (NiO), zinc oxide (ZnO), ferric oxide (Fe_2O_3) was mixed in a muller for 1 hour. The resulting powder was then screened through a 20 mesh screen. The powder was then treated in an oven at 350 degrees C. for 48 hours. The powder was then sintered at 1260 degrees C. in contact with air for 24 hours, and then cooled to room temperature over a period of 24 hours. Powders of different compositions were manufactured by varying the ratio of nickel oxide and zinc oxide in accordance with the relationship $NiOxZnO-Fe_2O_4$ where x is varied between 0.3 and 1.0. Frequency range absorbances are specified for some of the compositions in the following table.

Table 1

COMPOSITION	(X)	FREQUENCY RANGE (mHz)	%ABSORBANCE
$NiOZnOFe_2O_4$	.3	55 - 105	89
$NiOZnOFe_3O_4$	.6	145 - 1040	66
$NiOZnOFe_2O_4$	.9	530 - 2750	105

EXAMPLE 2

Preparation of Ferrite by Hydroxide Gel Process

.148 moles of $FeCl_3$ was dissolved in 50ml distilled water then precipitated with 150ml of 1M NaOH. .037 moles of $FeCl_2:4H_2O$ was dissolved in 50 ml distilled water then precipitated with 25ml of 1M NaOH. .0185Moles $CaCl_2$ was dissolved in 50ml distilled water and precipitated with 25ml of 1M NaOH. .0185 moles $ZnCl_2$ was dissolved in 50ml distilled water and precipitated with 25ml of 1M NaOH. All four precipitated solutions were added together in a large beaker and mixed vigorously for four min. in an industrial blender. The resulting gel was heated at 90 degrees C for 6 hours. O_2 was bubbled through the solution for the entire 6 hours.

EXAMPLE 3

Preparation of Ferrite by Hydroxide Gel Process

.148 moles of $FeCl_3$ was dissolved in 50 ml distilled water then precipitated with 150ml of 1M NaOH. .037 moles of $FeCl_2:4H_2O$ was dissolved in 50ml distilled water then precipitated with 25ml of 1M NaOH. .037 Moles $MnCl_2$ was dissolved in 50ml distilled water and precipitated with 25ml of 1M NaOH. All three precipitated solutions were added together in a large beaker and mixed vigorously in a blender for four min. The resulting gel was heated at 90 degrees C for 6 hours. O_2 was bubbled through the solution for the entire 6 hours.

EXAMPLE 4

Preparation of Ferrite by Hydroxide Gel Process

.148 moles of $FeCl_3$ was dissolved in 50ml distilled water then precipitated with 100ml of 0.1M LiOH. .037 moles of $FeCl_2:4H_2O$ was dissolved in 50ml distilled water then precipitated with 25ml 0.1MLiOH. Both precipitated solutions were added together in a large beaker and mixed vigorously for four min. The resulting gel was heated at 90 degrees C for 6 hours. 0_2 was bubbled through the solution for the entire 6 hours.

EXAMPLE 5

Preparation of Ferrites from Hydroxide Gels

A reactor provided with a heat exchange coil and a radial stirrer, was fed with 3600 ml of ferrous sulphate solution having a concentration of 40 g/liter. Subsequently, 290 ml of ammonia solution (200 g/liter of NH_3) were added thereto, while stirring at 100 rpm. Such stirring was carried on throughout the first step. Air was blown into the reactor at a flow rate of 100 cc/hr. and the temperature was kept at about 30 Deg. C by cooling the heat exchange coil with water. The first step of the reaction was concluded when the pH value decreased to 3.5 and the platinum electrode, with respect to the calomel electrode, indicated +110mV. This occurred about 7 hours after the beginning of the flowing in of air.

The analysis of the slurry was as follows:

Fe++ = 11.1g/liter; Fe = 37.1 g/liter.

160 ml of a ferrous sulphate solution (63.5 g/liter of Fe++) were admixed with the slurry. After this adjustment, the analysis of the slurry was as follows: Fe++ = 13.1 g/liter; Fe = 38.5 g/liter, the FeII/FeIII ratio being 0.52.

The reactor was fed with 155 ml of an ammonia solution (195 g/liter of NH_3) with stirring at 110 rpm. This stirring was continual throughout the second step. The

temperature was brought to 90 degrees C. by conveying steam into the heat exchange coil, and the temperature was kept constant by means of a thermostat. During the reaction the pH value decreased from 8 to about 6.5. The second step of the reaction was terminated when the redox potential rose from -700 to about -450 mV. This occurred about 3 hours from the beginning of the heating. At the end, the ferrous iron present as $Fe(OH)_2$ was 0.34 g//liter of FeII. The slurry was acidified to a pH value = 4 to remove ferrous hydroxide. The magnetic particles, once filtered, washed and dried, exhibited the following characteristics:

Morphology	Cubic
Average Diameter	
d10	0.182
Numerical variancy	
Coefficient	22.0%
Mg content	0.04%
S content	0.61%
Specific surface	6.52 m^2/g
magnetization 5680 G/domain	

EXAMPLE 6

Preparation of Oleic Acid Coated Magnetic Particles

Wave absorbing magnetic particles, coated with oleic acid were prepared using the ferrites prepared in Examples 1-5.

The ferrite powder is dispersed in a beaker with approximately 1500 cc distilled water, adjusted to a concentration of approximately 10 wt % and stirred with a paddle stirrer for about 5 minutes. The beaker containing the ferrite slurry is then placed onto a permanent magnet, separating the wave absorbing magnetic particle from the aqueous salt waste solution. After resting the slurry on the magnet for 5 minutes, the aqueous salt solution is decanted. The precipitate is then resuspended by agitation in an additional 1500 cc of fresh distilled

water. After the final water wash is decanted, the particles are suspended in acetone and the above washing procedure is repeated 5 times. The particles are then washed with hexane a total of five times each in the above manner.

Oleic acid is added to the magnetic particle/hexane slurry in a ratio of 2:1 oleic acid:dry particle. The mixture is adjusted to 15% total solids with hexane and milled overnight on a mechanical jar roller in a glass jar half filled with 3mm stainless steel balls.

The samples were labeled 1-5 to correspond to the ferrites prepared in Examples 1-5.

EXAMPLE 7

Preparation of Phenyl Lipid

A. Synthesis of a m-isophthalic acid based phenyl lipid.

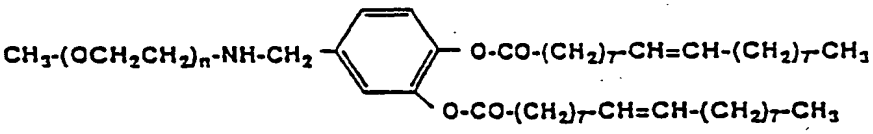
The starting material for this synthesis if 5-Aminoisophthalic acid. The 5-aminoisophthalic acid is not soluble in dioxane alone. It is soluble in a mixture of dioxane and triethylene glycol. 5-aminoisophthalic acid (145 mg) was dissolved in 5 ml. of dioxane and 2 ml. of triethylene glycol, and the pH was adjusted to 10 with NaOH. Methoxypolyoxyethylene imidazoly carbonyl, average mol. wt. 5,000 from Sigma (2.0g) was dissolved in 2ml of H_2O, 1.0ml of 1N Na_2CO_3, and 2.0 ml of triethylene glycol. This solution was added to the 5-aminoisophthalic acid solution and stirred for 36 hours at room temperature. The reaction mixture was then dialyzed overnight against 2 liters of H_2O. The dialyzed reaction mixture was mixed with 100ml of pyridine and the liquids removed via rotary evaporation. The resulting yellow oil was placed in the refrigerator. After several days a white precipitate formed. The precipitate contains both coupled and uncoupled PEG.

Oleyl alcohol can be coupled to the above isophthalic

acid derivative using thionyl chloride. The thionyl chloride can be used to activate the oleyl alcohol for ester formation with the carboxyl groups of the isophthalate. See. Fig. 2.

B. Synthesis of ortho phenyl lipids

The ortho analog of the phenyl lipids can be synthesized starting with either 3,4 dihydroybenzaldehyde or 3,4 dihydroxybenzoic acid. The aldehyde group can be coupled to an amino group by forming the Schiff's base and then reducing it with $NaBH_4$. Oleic acid could then be coupled to the hydroxyl groups using thionyl chloride to provide:



3,4 dihydroxybenzolic acid could be coupled through its carboxyl group to amino-terminated PEG using dicyclohexyl carbodiimide. Oleic acid could then be coupled as above.

Since both amino and carboxyl PEG derivatives as well as both oleic acid and oleylamine are available, the PEG and oleic acid groups can be easily interchanged in the above compounds.

EXAMPLE 8

Preparation of Magnetic Liposomes Using Phosphatidyl Choline

10 grams of each of the oleic acid coated ferrite as prepared in Example 6 were dispersed in 100 cc hexane. The phospholipid was absorbed onto the particle by dissolving phosphatidyl choline (Sigma, P-3644, L-2 lecithin, 45% PC) into hexane with heating to create a 15% solution. The PC/hexane solution was combined with the magnetic particles/hexane solution at a ratio of pure

phosphatidyl choline:oleic acid equal to 1:2 weight percent.

The solution was mixed in a glass jar (without media) on a jar roller for two hours. 50 cc of distilled water were added to the jar and mixing was continued for an additional 1 hour. The jar and its contents were then transferred to an ultrasonic bath and treated by ultrasound for an additional 30-60 minutes.

The slurry was transferred to a 200 cc beaker and heated on a hot plate to 100 dcg C for 10 min. From .05 to 50 grams of triton x-114 (Union Carbide) was added to disperse the lipidized ferrite in water. A ratio of triton X114:lipid particle equal to 1:6 weight percent was the optimum level for the dispersion. The dispersion was mixed on a laboratory vortex mixer for 2 minutes and placed in an ultrasonic bath (Branson 1200, VWR) for two hours. The final dispersion was adjusted to 0.2% TS (2mg/ml). Particles were measured on a Nycomp laser particle size analyzer and were found to be approximately 200 nm in diameter.

EXAMPLE 9

Preparation of Magnetic Liposomes using Phenyl Lipid

Samples were prepared using particles from Examples 1-5 exactly as described in Example 8 except that phenyl lipids prepared in Example #7 was used in place of PC.

Samples were labeled for later i.d. 6-10 to correspond with the particles as prepared in Examples 1-5. Samples were measured for particle size on a nycomp particle analyzer and found to be approximately 200 nm in diameter.

EXAMPLE 10

Preparation of MDCK Cell Cultures

Upon the arrival, ampules of CCL34, MDCK cells (NBL-2 canine kidney) from ATCC, are quickly thawed. Using a sterile Pasteur pipette the contents of the ampule are transferred to a flask containing at least 10 volumes of

culture medium (Eagles MEM) previously adjusted to pH 7.4. The cells are incubated for 24 hours, the media is withdrawn, discarded and replaced. Cells are incubated at 36.5 degrees C. in a CO_2 incubator for approximately 7 days. Another medium change may be necessary if indicated by a drop in pH or high cell concentration.

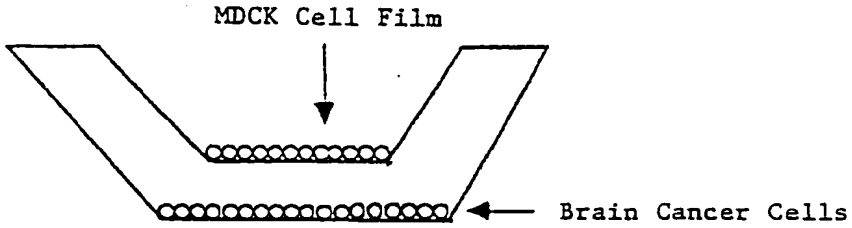
Cells are transferred during log phase, once confluence has been reached. The procedure is as follows: The media is withdrawn and discarded. A PBSA (5ml/25cm 2) prewash is added to the flask opposite the cell monolayer. To avoid disruption the cells are rinsed and the solution discarded. Next, 3 ml/25 cm 2 trypsin is added to the flask (opposite of cells). The flask is turned to expose the cells to the trypsin for 15-30 seconds, then the trypsin is discarded making sure the monolayer is not detached. The cells are incubated until the monolayer will slide down the flask surface when tipped. (Approximately 5-15 min.) MEM medium is used to disperse the cells by repeated pipetting. Cells are diluted to 10-100 cells/ml and seeded in transwells as follows: Costar 6 well transwell-COL(3418) with pore size of 3.0 micron and well and 1.5ml of culture (media and cells) are added to the inside of the transwell. The wells are covered and incubated until the monolayer is established on the membrane. The cell cultures thus prepared were used for all further experiments.

EXAMPLE 11

Ferrites were prepared as described in Examples 1-5, coated with oleic acid as in Example #6 and treated with a second layer of phenyl lipid as described in Example #7.

A culture of MDCK cells were prepared as described in Example #10. The lipid coated ferrites and uncoated (bare) ferrite controls were put in contact with the MDCK cells grown above a colony of rat brain cancer cells

(neuroblastoma) as detailed in the figure below.



The sample was allowed to incubate at room temperature for a period of 1 hour, then exposed to a frequency of 20000 mHz for 3 minutes. None of the bare ferrite were permeable to the endothelial cell (MDCK) membrane and had no effect on the cancer cell colony.

Ferrites as prepared in Example 1, 2, 3 and 4 rapidly heated upon exposure to the EM wave and all the brain cells in the culture were killed.

Ferrites as prepared in Sample #5 were able to cross the endothelial cell barrier, however, because they are all iron, do not absorb EM waves and had no effect on the neuroblastoma cells.

CLAIMS

1. A coated magnetic or superparamagnetic responsive particle comprising:

a. a magnetic core particle comprising a magnetically-responsive metal, metal alloy or metal oxide; and

b. an organo-metallic polymer coating covalently bonded to or absorbed onto said particle wherein the bonding or adsorbtion does not depend upon the presence of hydroxy functionality on the surface of said particle, and wherein the organo-metallic polymer coating preferably has functional groups selected from the group consisting of amino, carboxyl, hydroxyl, sulfate, phosphote, vinyl, nitrate, aldehyde, epoxy, succinamine, anhydride, cyanate, and thiol groups, and is capable of binding at least one type of bioaffinity adsorbent, preferably selected from the group consisting of antibodies, antigens, enzymes and specific binding proteins.

2. A coated magnetically responsive particle of claim 1, wherein the magnetic core particle comprises a metal, metal alloy or metal oxide selected from the group consisting of iron, magnetite, iron magnesium oxide, iron manganese oxide, iron cobalt oxide, iron nickel oxide, iron zinc oxide and iron copper oxide, preferably containing a particle size of from about 0.003 to about 1.5 microns in diameter, wherein the organo-metallic polymer is preferably formed from monomers which are coordinate complexes of organic ligands and a metal selected from the group consisting of: titanium, zirconium, hafnium, vanadium, tanatalum, niobium, tin, antimony, zinc, cadmium, manganese, tellerium, rhenium, aluminum, gallium, germanium and iridium, or wherein the organo-metallic polymer is preferably an organo-titanium polymer selected from the group consisting of: titanium-tetra-isopropoxide, amino-hexyl-titanium-triisopropoxide,

amino-propyl-titanium-triisopropoxide and carboxyl-hexyl-titanium triisopropoxide.

3. A method of measuring analytes in a sample comprising the steps of:

a. contacting a sample containing an unknown concentration of the analyte with a known amount of a labeled analyte in the presence of magnetic particles comprising:

(i) a magnetic core particle comprising a magnetically responsive metal, metal alloy or metal oxide; and

(ii) an organo-metallic polymer coating covalently bonded to or adsorbed onto said particle wherein the bonding or adsorbtion does not depend upon the presence of hydroxy functionality on the surface particles, and wherein said organo-metallic coating has a bioaffinity adsorbent covalently coupled thereto, said bioaffinity adsorbent is capable of binding to or interacting with both the unlabeled and the labeled analyte;

b. maintaining the mixture in step (a) under conditions sufficient for said binding or interaction to occur;

c. magnetically separating the magnetic particles; and

d. measuring the amount of label associated with the magnetic particles and determining the concentration of analyte in solution.

4. The method of claim 3 wherein the analyte is preferably selected from the group consisting of: antibodies, antigens, haptens, enzymes, apoenzymes, enzymatic substrates, enzymatic inhibitors, cofactors, nucleic acids, binding proteins, carrier proteins, compounds bound by binding proteins, compounds bound by carrier proteins, lectins, monosaccharides, polysaccharides, hormones, receptors, repressors and

inducers; wherein the magnetic core particle preferably comprises a metal, metal alloy or metal oxide selected from the group consisting of: iron, magnetite, iron magnesium oxide, iron manganese oxide, iron cobalt oxide, iron nickel oxide, iron zinc oxide and iron copper oxide, and preferably has a particle size of from about 0.003 to about 1.5 microns in diameter; wherein the organo-metallic polymer coating is preferably formed from monomers which are coordinate complexes of organic ligands and a metal selected from the group consisting of: titanium, zirconium, hafnium, vanadium, tantalum, niobium, tin, antimony, zinc, cadmium, manganese, tellerium, rhenium, aluminum, gallium, germanium and iridium; wherein the organo-metallic polymer is more preferably an organo-titanium polymer selected from the group consisting of: titanium-tetra-isopropoxide, amino-hexyl-titanium triisopropoxide, amino-propyl-titanium isopropoxide and carboxyl-hexyl-titanium triisopropoxide; wherein the magnetically responsive particle is preferably superparamagnetic; wherein the bioaffinity adsorbent is preferably selected from the group consisting of: antibodies, antigens, haptens, enzymes, apoenzymes, enzymatic substrates, enzymatic inhibitors, cofactors, nucleic acids, binding proteins, carrier proteins, compounds bound by binding proteins, compounds bound by carrier proteins, lectins, monosaccharides, polysaccharides, hormones, receptors, repressors and inducers; and wherein the labeled analyte is preferably marked with a label selected from the group consisting of: radioisotopes, fluorescent compounds, enzymes and chemiluminescent compounds.

5. A method for preparing inorganic oxides of substantially uniform particle size distribution comprising contacting aqueous solutions of an inorganic salt and an inorganic base across a porous membrane wherein the membrane contains a plurality of pores which

allows for precipitation of a substantially mono-dispersed size inorganic oxide particles on one side of the membrane and precipitation of a salt of the corresponding base on a second side of the membrane.

6. The method of claim 5 wherein the particle size diameter is preferably 20, 50, 80 or 100A; and wherein the particle size distribution is preferably +/- 10%; wherein the inorganic salt is preferably of the formula MY, wherein M is selected from the group consisting of Fe, Co, Ni, Zn, Mn, Mg, Ca, Ba, Sr, Cd, Hg, Al, B, Sc, Ga, V In, and mixtures thereof; and wherein the inorganic salt is of the formula MY, and Y is preferably selected from the group consisting of Cl, Br, I, SO_4, NO_3, PO_4 and mixtures thereof; and wherein the inorganic base is preferably selected from the group consisting of NH_4OH, KOH, LiOH, NaOH, CsOH, RbOH and mixtures thereof; and wherein the substantially mono-dispersed precipitated inorganic oxide particle size is preferably from about 5-1000A in diameter; and wherein the substantially mono-dispersed precipitated inorganic oxide particle is of the formula M_3O_4 wherein M is preferably selected from the group consisting of Fe, Co, Ni, Zn, Mn, Mg, Ca, Ba, Sr, Cd, Hg, Al, B, Sc, Ga, V, In and mixtures thereof; and wherein the substantially mono-dispersed precipitated inorganic oxide particle is preferably Fe_3O_4; and wherein the size of the precipitated inorganic oxide particle is preferably increased by selecting an inorganic base with a relatively rapid dissociation constant; and wherein the size of the precipitated inorganic oxide particle is preferably reduced by selection of an inorganic base with a relatively slow dissociation constant; and wherein the size of the precipitated inorganic oxide particles is further controlled by varying the pore size of the membrane, the temperature of the inorganic salt and inorganic base solutions, and the concentration of the aqueous inorganic salt solution; and wherein the

concentration of the aqueous inorganic salt solution is preferably about 1-3%wt; and wherein the size of the precipitated particles is controlled by adjusting the concentration of the aqueous inorganic base; and wherein the concentration of the aqueous solution of inorganic base is preferably about 2-4%wt; and wherein the aqueous inorganic salt solution and the aqueous inorganic base are preferably allowed to remain in contact across said membrane for a period of about 40-80 hours; and wherein said membrane is preferably selected from material consisting of cellulose polymer, a fluropolymer, a chlorinated olefin polymer, and a polyamide; and wherein the pore size of the membrane as measured by the molecular weight cut-off is preferably adjusted between 1000 and 500,000.

7. A controllably degradable aggregate cluster comprising a cluster of inorganic oxides of substantially mono-dispersed particle size which are coated with a functionalized organic moiety wherein the cluster is bonded together by chemical, complex, or ionic coupling between the functional groups of said organic moiety.

8. The controllably degradable aggregate cluster of claim 7 wherein the functionalized organic moiety is preferably an organo-metallic polymer; and wherein the organo-metallic polymer coatings are formed from organo-metallic monomers selected from the group consisting of: amino-hexyl-titanium triisopropoxide, amino-propyl-titanium triisopropoxide and carboxy-hexyl-titanium triisopropoxide; and wherein the aggregate cluster is preferably superparamagnetic and the individual particles are non-magnetic.

9. A controllable degradable aggregate bead cluster which comprises:

a cluster of inorganic oxide particles of substantially mono-dispersed particle size associated with a macromolecular species, characterized in that said

particles are encapsulated by the macromolecular species forming a bead, the macromolecular species containing organic functionality to link the beads together forming controllably degradable chemical, complex, or ionic bonds.

10. The controllably degradable aggregate bead cluster of claim 9 wherein the macromolecular species is selected from the group consisting of polystyrene, poly(vinyl chloride) and polyurethane; and wherein the bead is preferably formed by surrounding the particles with a difunctional organic monomer, one functionality of the monomer adsorbed onto or covalently bound to the particles, one functionality covalently bonded as between monomers forming macromolecular encapsulation; and wherein the aggregate bead cluster is preferably superparamagnetic, and the individual beads are non-magnetic.

11. A method for determining the concentration of a ligate in solution which comprises:

a. providing a substantially mono-dispersed inorganic oxide particle of claim 5 wherein said particles are non-magnetic;

b. coating said particles with an organo-metallic polymer coating which is adsorbed onto or covalently bound to the particle and which is functionalized to covalently bind to a ligand moiety having specific affinity for the ligate to be measured;

c. covalently binding said ligand moiety to the particle;

d. reacting the product in step (c) with a solution containing the ligate to be measured to form a ligand/ligate magnetic complex;

e. relating the magnetic response of the product in step (d) to the concentration of the ligate causing the complexation.

12. The method of claim 30 wherein the ligand is an antibody and the antibody is preferably selected from the

group consisting of anti-thyroxine, anti-triiodothyronine, anti-thyroid stimulating hormone, anti-thyroid binding globulin, anti-thyroglobulin, anti-digoxin, anti-cortisol, anti-insulin, anti-theophylline, anti-vitamin B-12, anti-folate, anti-ferritin, anti-human chorionic gonadotropin, anti-follicle stimulating hormone, anti-progesterone, anti-testosterone, anti-estriol, anti-estradiol, anti-prolactin, anti-human placental lactogen, anti-gastrin and anti-human growth hormone antibodies; and wherein the ligate is preferably selected from the group consisting of hormones, peptides, pharmacological agents, vitamins, cofactors, hematolgical substances, virus antigens, nucleic acids and nucleotides; and wherein the ligate is more preferably selected from the group consisting of thyroxine, theophylline, vitamin B-12, triiodothyronine, and thyroid stimulating hormone, and the ligand is selected from the group consisting of anti-theophylline anti-body, vitamin B-12 binding protein, and anti-thyroid stimulating hormone anti-body.

13. A method for determining the concentration of a metal in solution which comprises:

a. providing a substantially mono-dispersed inorganic oxide particle of claim 1 wherein said particles are non-magnetic;

b. coating said particles with an organo-metallic polymer coating which is adsorbed onto or covalently bound to the particle and which is functionalized to covalently bind to an organic moiety having specific affinity for the metal to be measured;

c. covalently binding said organic moiety to the particle;

d. reacting the product in step (c) with a solution containing the metal to be measured to form a magnetic complex;

e. relating the magnetic response of the product in step (d) to the concentration of the metal causing the

complexation.

14. The method of claim 13 wherein the organic moiety having specific affinity for a metal to be measured is preferably 2,3-dihydroxy-5-benzoic acid; and wherein the metal to be measured is preferably selected from the group consisting of Tu and Mo; and wherein the organic moiety having specific affinity for the metal to be measured is preferably 2,3-dithio-5-benzoic acid and the metal to be measured is Mo.

15. A liposome composition comprising a substantially uniform size inorganic core coated with an amphipathic organic compound and further coated with a second amphipathic vesicle forming lipid.

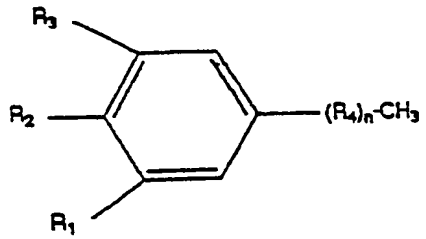
16. The liposome composition of claim 15 wherein the inorganic core is preferably selected from the group consisting of Fe_3O_4, Fe_2O_3, Al_2O_3, TiO_2, ZnO, FeO and Fe; and wherein the inorganic core is preferably a substantially uniform sub 100 nm diameter inorganic oxide; and wherein the amphipathic organic compound is preferably a fatty acid selected from the group consisting of oleic, linoleic, linolenic, palmitic, myristic and arachidonic acid; and wherein the vesicle forming lipid is preferably selected from the group consisting of phospholipids, sterol lipids and glycolipids; and wherein the phospholipid is preferably selected from the group consisting of phosphatidylcholine, phosphatidic acid and phosphatidylinositol.

17. A liposome composition for use in delivering a compound via the bloodstream comprising a substantially uniform size inorganic core coated with an amphipathic organic compound and further coated with 1-20 mole percent of an amphipathic vesicle-forming lipid derivatized with a hydrophilic polymer, and containing the compound in liposome-entrapped form.

18. The composition of claim 17 wherein the hydrophillic polymer is preferably selected from the group

consisting of poly(ethylene oxide), poly(propylene oxide) and poly(vinyl alcohol); and wherein the liposomes preferably have a selected average size in the size range between about 5 and 5000 nanometers; and wherein the hydrophilic polymer preferably has a molecular weight between about 1,000 to 5,000 daltons; and wherein the vesicle forming lipid is preferably selected from the group consisting of phospholipids, sterol lipids, and glycolipids; and wherein the phospholipid is preferably derivatized with poly(ethylene oxide); and wherein the phospholipid is preferably phosphatidylethanolamine and the poly(ethylene oxide) is coupled to the phosphatidylethanolamine through a lipid amine group.

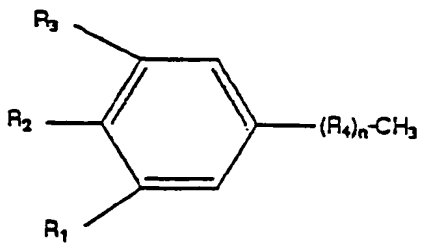
19. A synthetic vesicle forming phenyl lipid compound having the structural formula:



wherein two of R_1, R_2 and R_3 represent saturated or unsaturated straight-chain or branched chain alkyl or acyl groups, the other being hydrogen, and R_4 is an alkylene oxide or vinyl alcohol repeat unit and n varies from 0 to about 200.

20. A liposome composition for use in delivering a compound via the bloodstream containing the compound in liposome entrapped form comprising a substantially uniform size inorganic core coated with an amphipathic compound and further coated with 1-20 mole percent of an amphipathic vesicle-forming phenyl lipid having the

formula:



wherein two of R_1, R_2 and R_3 represent saturated or unsaturated straight-chain or branched chain alkyl or acyl groups, the other being hydrogen, and R_4 is an alkylene oxide or vinyl alcohol repeat unit and n varies from 0 to about 200.

21. The composition of claim 20 wherein the alkylene oxide repeat unit is preferably selected from the group consisting of ethylene oxide and propylene oxide; and wherein the branched chain alkyl or acyl groups are organic radicals preferably derived from the group consisting of oleic acid, stearic acid, linoleic acid, linolenic acid, palmitic acid, myristic acid, and arachidonic acid.

22. A method for preparing a substantially uniform size inorganic core liposome composition comprising the steps of preparing a substantially uniform size organic oxide particles, coating said particle with an amphipathic organic compound wherein the organic compound is adsorbed or coordinated onto the surface of the inorganic oxide, and associating said coated particle with an amphipathic vesicle forming lipid.

23. The method of claim 22 wherein the substantially uniform size inorganic oxide particle is preferably prepared by contacting aqueous solutions of an organic salt and an inorganic base across a porous membrane wherein the membrane contains a plurality of pores which allows for precipitation of a substantially uniform size inorganic oxide particle on one side of the membrane and precipitation of a salt of the corresponding base on a

second side of the membrane; and wherein the substantially monodispersed precipitated inorganic particle size is preferably from about 5-1000A in diameter; and wherein the substantially monodispersed precipitated inorganic oxide particle is preferably Fe_3O_4.

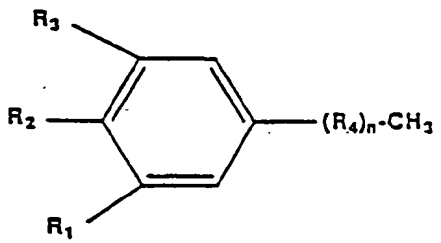
24. A composition comprising a wave absorbing magnetic core particle coated with an amphipathic organic compound and further coated with a second amphipathic vesicle forming lipid.

25. The composition of claim 24 wherein the wave absorbing magnetic core particle is a ferrite material of the formula $M_2(+3)M(+2)O_4$, wherein the M(+3) is preferably selected from the group consisting of Al, Cr and Fe, and M(+2) is preferably selected from the group consisting of Fe, Ni, Co, Zn, Ze, Ca, Ba, Mg, Ga, Gd, Mn and Cd; and wherein the ferrite material is preferably mixed with LiO, NaO, KO, Fe_2O_3 or Fe_3O_4; and wherein the wave absorbing magnetic core particle is preferably a substantially uniform sub 100 nm diameter ferrite particle; and wherein the amphipathic organic compound is preferably a fatty acid selected from the group consisting of oleic, linoleic, linolenic, palmitic, myristic and arachidonic acid; and wherein the vesicle forming lipid is preferably selected from the group consisting of phospholipids, sterol lipids and glycolipids; and wherein the phospholipid is preferably selected from the group consisting of phosphatidylcholine, phosphatidic acid, phosphatidylinositol, and phosphatidal ethonalamine.

26. A liposome composition for use in delivering a compound via the bloodstream comprising a wave absorbing magnetic core coated with an amphipathic organic compound and further coated with 1-20 mole percent of an amphipathic vesicle-forming lipid derivatized with a hydrophilic polymer, and containing the compound in liposome-entrapped form.

27. The composition of claim 26 wherein the hydrophillic polymer is preferably selected from the group consisting of poly(ethylene oxide), poly(propylene oxide) and poly(vinyl alcohol); and wherein the liposomes preferably have a selected average size in the size range between about 5 and 5000 nanometers; and wherein the hydrophilic polymer preferably has a molecular weight between about 1,000 to 5,000 daltons; and wherein the vesicle forming lipid is preferably selected from the group consisting of phospholipids, sterol lipids, and glycolipids; and wherein the phospholipid is preferably derivatized with poly(ethylene oxide); and wherein the phospholipid is preferably phosphatidylethanolamine and the poly(ethylene oxide) is coupled to the phosphatidylethanolamine through a lipid amine group.

28. A liposome composition for use in delivering a compound via the bloodstream containing the compound in liposome entrapped form comprising a wave absorbing magnetic core coated with an amphipathic compound and further coated with 1-20 mole percent of an amphipathic vesicle-forming phenyl lipid having the formula:



wherein two of R_1, R_2 and R_3 represent saturated or unsaturated straight-chain or branched chain alkyl or acyl groups, the other being hydrogen, and R_4 is an alkylene oxide or vinyl alcohol repeat unit and n varies from 0 to about 200.

29. The composition of claim 28 wherein the alkylene oxide repeat unit is preferably selected from the group consisting of ethylene oxide and propylene oxide; and wherein the branched chain alkyl or acyl groups are

organic radicals preferably derived from the group consisting of oleic acid, stearic acid, linoleic acid, linolenic acid, palmitic acid, myristic acid, and arachidonic acid.

30. A process for the preparation of substantially uniform size oxides of the formula $M_2(+3)M(+2)O_4$ comprising:

supplying separate aqueous metal solutions of M(+3) and M(+2);

adding alkali to said aqueous solutions and precipitating the corresponding metal hydroxide; and

mixing the metal hydroxide precipitates in solution together and heating to dehydrate, wherein the dehydration temperature, time of dehydration, and concentration of oxygen or air passed through the solution are adjusted to control the particle size of the oxide particle produced.

31. The process of claim 19 wherein M(+3) is preferably selected from the group consisting of Al, Cr and Fe, and M(+2) is preferably selected from the group consisting of Fe, Ni, Co, Zn, Ze, Ca, Ba, Mg, Ga, Gd, Mn and Cd; and wherein the dehydration temperature is preferably 100°C and the dehydration temperature is 6 hours.

32. A method for preparing a wave absorbing magnetic core liposome composition comprising the steps of supplying wave absorbing magnetic core particles, coating said particles with an amphipathic organic compound, preferably an organometallic compound, wherein the organic compound is adsorbed or coordinated onto the surface of the said particle, and associating said coated particle with an amphipathic vesicle forming lipid.

33. The process for the treatment of cancer cells or infectious disease organisms by application of external electromagnetic energy capable of the generation of heat in intracellular particles to induce selective thermal death of cancer cells comprising:

placing within the patient wave absorbing magnetic core particles coated with an amphipathic organic compound and further coated with a second amphipathic vesicle forming lipid,

absorbing said coated wave absorbing magnetic core particle intracellulary into the cancer cells,

subjecting the patient to an alternating electromagnetic field to inductively heat the magnetic core particle and thereby the cancer cells, and

continuing the inductive heating of said magnetic core particle to attain an increase in intracellular temperature to selectively kill either the cancer cells or said organism.

34. The process of claim 33 wherein the magnetic particles are ferrites, whose oxide component is of the formula $M_2(+3)MO_4$, wherein M(+3) is preferably selected from the group consisting of Al, Cr and Fe, and M is preferably selected from the group consisting of Fe, Ni, Co, Zn, Ze, Ca, Ba, Mg, Ga, Gd, Mn and Cd; and wherein the wave absorbing magnetic core is preferably a substantially uniform size wave absorbing magnetic core particle preferably in the range of from about 1 to 50,000 nm in diameter.

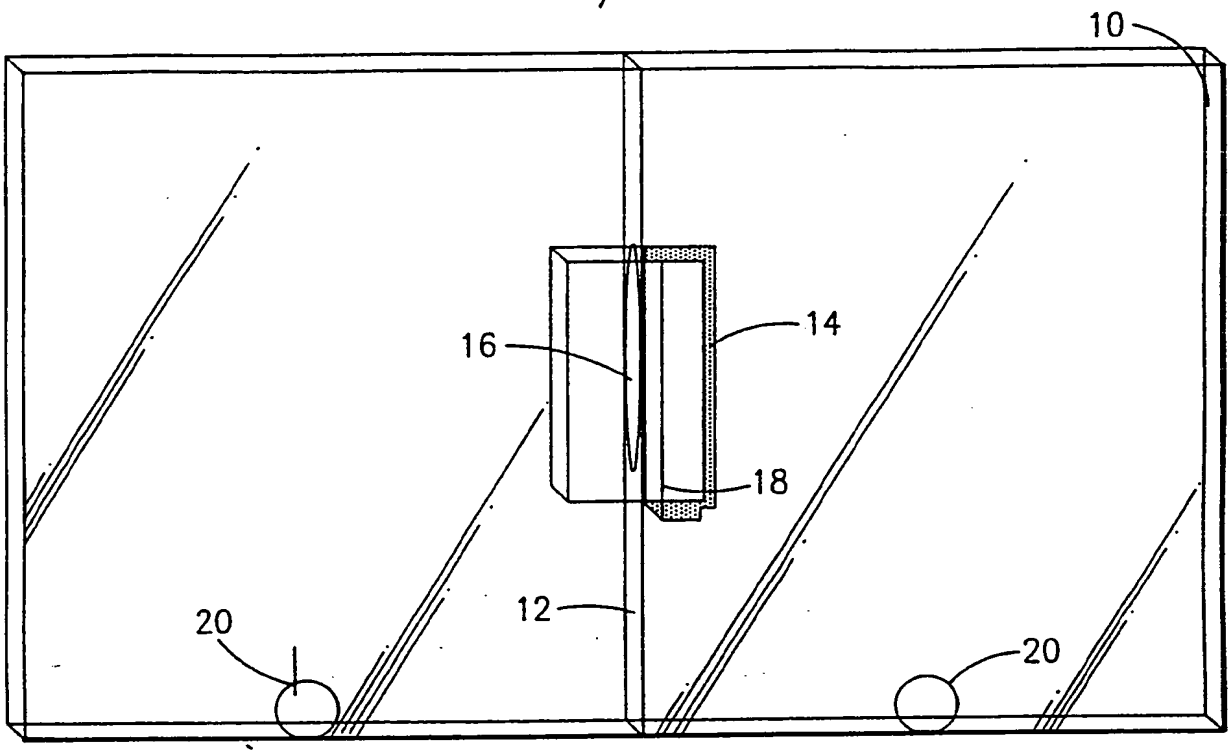


FIG.1

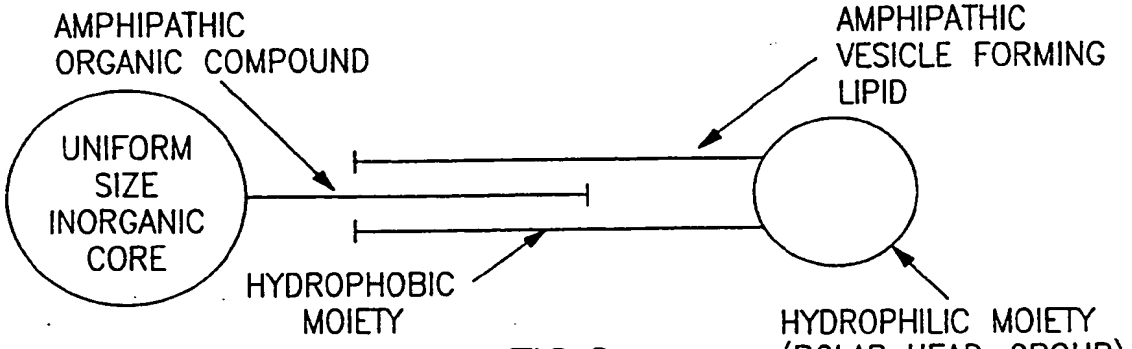


FIG.2

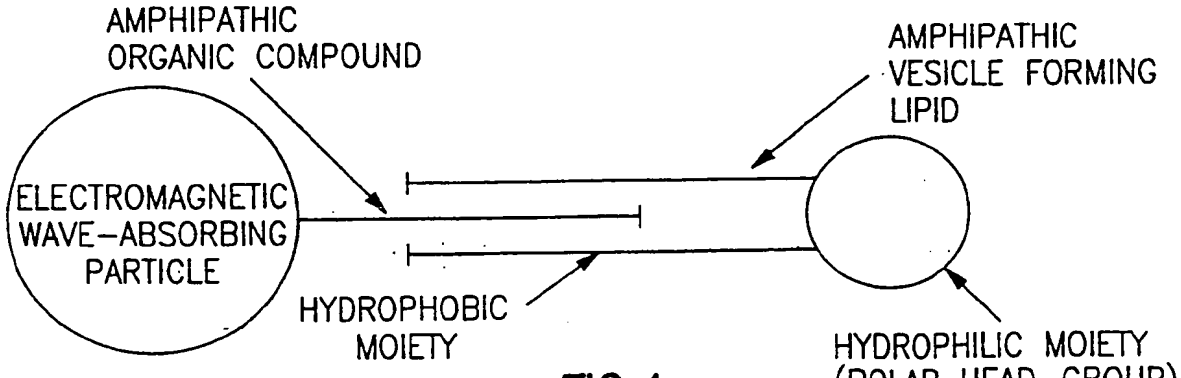


FIG.4

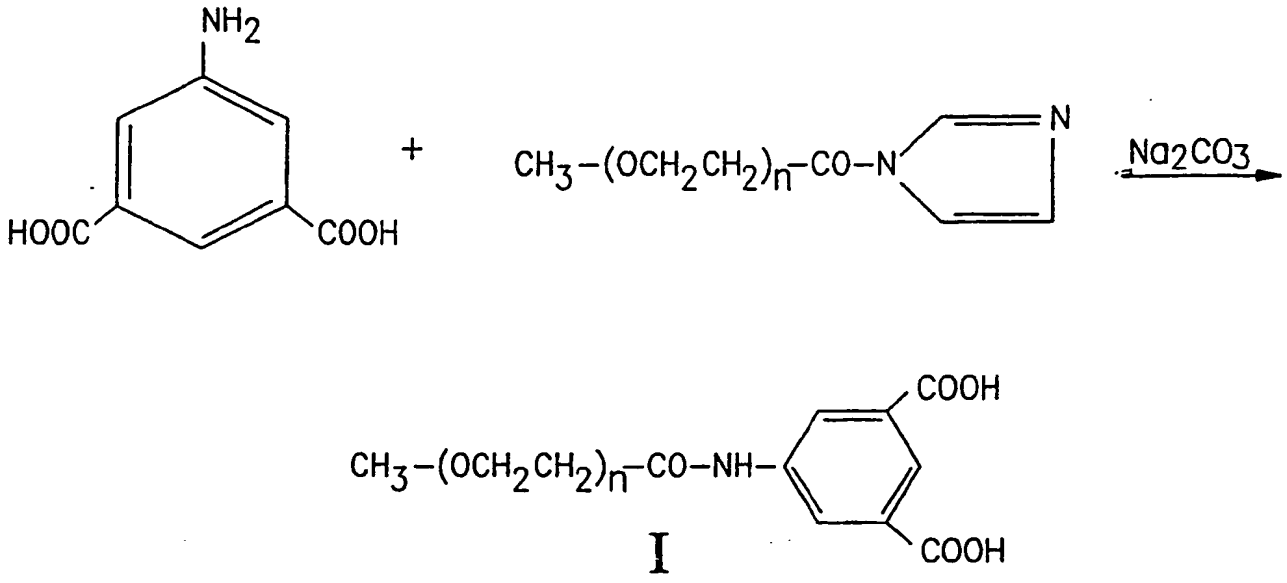


FIG.3

INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 93/05595

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 H01F1/06 G01N33/543 C12N11/00 G01N33/58 C01G1/02
A61K9/127 A61K49/00 C07C69/58 C08G65/26

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 G01N C01G A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	EP,A,0 546 939 (MOLECULAR BIOQUEST, INC.) 16 June 1993 see the whole document ---	1-4,7-12
X	WO,A,91 09678 (OMNI QUEST CORPORATION) 11 July 1991 see the whole document ---	1-4,11, 12
A	EP,A,0 125 995 (ADVANCED MAGNETICS, INC.) 21 November 1984 & US,A,4 628 037 cited in the application ---	
A	WO,A,89 11335 (LIPOSOME TECHNOLOGY, INC.) 30 November 1989 & US,A,4 994 213 cited in the application ---	
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 October 1993	03. 11. 93

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	GRIFFITH, G

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INTERNATIONAL SEARCH REPORT

Inte onal Application No
PCT/US 93/05595

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US,A,4 675 173 (K. J. WIDDER) 23 June 1987 cited in the application	
A	US,A,4 945 049 (T. HAMAYA ET AL.) 31 July 1990 cited in the application	
A	US,A,5 071 076 (M. S. CHAGNON ET AL.) 10 December 1991 cited in the application	

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ional Application No
PCT/US 93/05595

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0546939	16-06-93	US-A-	5225282	06-07-93
		CA-A-	2084970	14-06-93
WO-A-9109678	11-07-91	EP-A-	0506880	07-10-92
		JP-T-	5502944	20-05-93
EP-A-0125995	21-11-84	US-A-	4554088	19-11-85
		CA-A,C	1254028	16-05-89
		DE-A-	3485332	23-01-92
		EP-A-	0357593	14-03-90
		JP-A-	60001564	07-01-85
		WO-A-	8806632	07-09-88
		US-A-	4628037	09-12-86
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		US-A-	4695393	22-09-87
		US-A-	4698302	06-10-87
		US-A-	4672040	09-06-87
US-A-4628037	09-12-86	US-A-	4554088	19-11-85
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WO-A-8911335	30-11-89	US-A-	4994213	19-02-91
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		AU-A-	3564589	12-12-89
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		WO-A-	8911335	30-11-89
US-A-4675173	23-06-87	US-A-	4849210	18-07-89
US-A-4945049	31-07-90	WO-A-	8901521	23-02-89

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int onal Application No
PCT/US 93/05595

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-5071076	10-12-91	EP-A- 0542829	26-05-93
		WO-A- 9202940	20-02-92

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